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14. ABSTRACT

The burden of fungal identification is challenging for military clinicians due to a lack of access to technology, expertise, and/or instrumentation. The unique military patient population, and exposure to rarely seen fungi, exacerbates the problem. New diagnostic methods suitable to the unique military environment are urgently needed. The objective of this proposal is to develop a rapid molecular diagnostic assay using a new PCR technology called LATE-PCR (Linear-After-The-Exponential Polymerase Chain Reaction). This study has three aims: 1) selection of targets suitable for a multiplex PCR reaction, 2) design of primers and probes for each class of fungus, all of which will be compatible in a single reaction, and 3) pilot testing on tissue specimens. PCR is a tried and tested molecular method with broad application, including FDA-approval. Reagents can be prepackaged and ruggedized instrumentation suitable for forward stations are available. The novelty of LATE-PCR is that it is a single tube reaction capable of multiplexing up to 32 targets (fungal species), which eliminates the need for applying a variety of diagnostic tests to an unknown fungal specimen.

15. SUBJECT TERMS
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INTRODUCTION: The burden of fungal identification is challenging for military clinicians due to a lack of access to technology, expertise, and/or instrumentation. The unique military patient population, and exposure to rarely seen fungi, exacerbates the problem. New diagnostic methods suitable to the unique military environment are urgently needed. The objective of this proposal is to develop a rapid molecular diagnostic assay using a new PCR technology called LATE-PCR (Linear-After-The-Exponential Polymerase Chain Reaction).

LATE-PCR stands for Linear-After-The-Exponential PCR [1, 2], and is a modified form of asymmetric PCR [3]. Asymmetric PCR functions by generating an excess of single strands due to an imbalance in primer ratios, which can be ≥10:1 of one primer over the other. This imbalance results in a short exponential amplification to increase the number of template strands, which is then followed by a switch to a linear amplification of one of the two strands to yield a large population of single strands. LATE-PCR utilizes more efficient primer and probe kinetics to produce the single-stranded amplification products, which offer a number of advantages over double stranded products produced in conventional PCR. Single strands enhance probe sensitivity due to the lack of binding competition from the complimentary strand, which is present in the reaction mix in conventional PCR, but not in LATE-PCR. Additionally, LATE-PCR amplicons can be quite long, on the order of 1000 base pairs or more, compared to shorter amplicons for traditional real-time PCR. This longer product allows easier multiplexing, resulting in the inclusion of more discriminating probes in a single reaction that ultimately can detect up to 35 targets in one tube and can enhance the discriminatory power over real time PCR by more than 10x [4, 5]. Finally, if needed, the single stranded product can be directly sequenced after the reaction without a cleanup step [6], which will work seamlessly with our existing database should a sequencing step be needed. The LATE-PCR strategy is further enhanced by additional technologies that include dedicated reagents and importantly, specific algorithms for primer and probe design, which maximizes reaction efficiency.

This study has three aims: 1) selection of targets suitable for a multiplex LATE-PCR reaction, 2) design primers and probes for each target, all of which will be compatible in a single reaction, and 3) pilot test assay on tissue specimens. We will use our sequencing data from the previous DOD award period to develop a LATE-PCR-based assay targeted to specific fungal infections of trauma patients. We will first identify the molecular targets using our database, design and test the LATE-PCR reaction, and finally, pilot test our assay on infected tissues from trauma patients with proven fungal infections. PCR is a tried and tested molecular method with broad application, including FDA-approval. Reagents can be prepackaged and ruggedized instrumentation suitable for forward clinics are available. The novelty of LATE-PCR is that it is a single tube reaction capable of multiplexing up to 32 targets (fungal species), which eliminates the need for applying a variety of diagnostic tests to an unknown fungal specimen.

BODY: This reporting period consists of the first annual report and covers 4 quarters of research, comprising the first year or a two-year award. We have completed Task 1, begun Task 2, and made substantial, although unexpected progress on Task 3 due to an opportunity to examine some clinical specimens.

TASK 1, MILESTONE 1. SELECTION OF TARGET SPECIES. (Months 1-4).
In the first reporting period, the proposed work consisted of establishing a priority list of fungi to be identified by our assay, and then in subsequent months we would identify the molecular targets to be included in the assay. As proposed in the study, we planned to identify infections caused by members of the *Aspergilli*, *Fusaria*, and Zygomycetes.

We first selected members of the Zygomycetes as they are particularly important for trauma patients and have selected target species based on frequency of recovery and have chose *Rhizopus*, *Mucor*, *Absidia*, and *Cunninghamella* as the genera to design our individual probes for. We next chose the second most difficult genus of fungi to identify, which are the fusaria. In selecting the important pathogenic species from this genus, we identified *Fusarium moniliforme*, *Fusarium oxysporum*, *Fusarium solani Fusarium incarnatum-equisetti*, *Fusarium chlamydosporum*, *Fusarium dimerum*, *Fusarium napiforme*, *Fusarium sporotrichoides*, and *Fusarium graminearum*. However, as in the Zygomycetes, the genus *Fusarium* can have many pathogenic members that cause infection, but only a few cause the majority of infections and current clinical mycology often reports identifications that reflect this, such as noting a *Fusarium* species was identified as an *F. solani* species complex. Consequently, we are targeting each of the *Fusarium* complexes in order to have a chance of detecting a broad spectrum of Fusarium species. The third group of fungi that we are working with are the *Aspergilli*. The most important pathogenic members of this genus are *A. fumigatus, A. terreus, A. flavus. A. niger*, which we will target. In discussing a diagnostic assay with clinicians, they have noted that discriminating *A. terreus* from the rest of the Aspergilli is important due to increased drug resistance of this species, hence we are incorporating this goal in the assay.

TASK 1, MILESTONE 2. IDENTIFICATION OF TARGET SEQUENCES. (Months 3-12).
Our target sequences were determined by aligning multiple sequences for multiple genes to identify the best region to target with our primers and probes. For the Zygomycetes, we found a region of about 130 bp over an alignment of ~1000 bp within the 28S ribosome gene. During the latter course of this task, we had an opportunity to work with some FFPE specimens that were suspected to contain fungal elements consistent with zygomycosis. We designed a number of test primers to see if we could amplify DNA from this region and were successful, which was confirmed by sequencing. Importantly, we controlled for human DNA as fixed specimens will predominantly contain human DNA. We found that we did not get any false positives, which was predicted from our alignments. We next looked at different sequences for the Fusaria and selected the elongation factor 1 alpha gene as a target with a strategy of designing clade-specific primers in probes, which will give is the best chance of detecting any *Fusarium* species, while still providing informative diagnostic information. For the *Aspergilli*, we have selected the ribosomal region containing the ITS sequence.

TASK 2, MILESTONE 1. DESIGN LATE-PCR PRIMERS AND PROBES. (Months 10-18).
We have recently started designing LATE-PCR primers and probes and have been performing test runs using pilot primers to insure that the regions are good targets. We will first test primers against multiple isolates and then add the probes once the primers are confirmed to work. This strategy will save money in the event of a need to redesign primers or probes, or choose alternate DNA targets.

TASK 3, MILESTONE 1. DEVELOPMENT OF TEMPLATE PREPARATION PROTOCOLS FOR CLINICAL SPECIMENS. (Months 18-24).

This task was originally scheduled for our second year, however, we had the opportunity to test our preparation methods on fixed tissue and fresh tissue. We utilized template preparation methods that have been used in our past publications on animal studies and found that they work well for tissue specimens as we obtained PCR products and sequencing confirmed that they were fungal. An important result for this milestone was the discovery that our template preparation method works on fixed tissue as well, with modification. We combined our laboratory method with a commercial FFPE extraction kit, and then used an automated DNA extractor to finish the extractor. We were encouraged to see all of our short primer pairs (amplicons ~100 bp) yielded PCR products. Because these all worked, and fixed specimens are notoriously difficult to amplify, we tried longer primers and were surprised to see that we were able to amplify products ~500 bp in length, which is extremely difficult to do with fixed tissue. This methodology was submitted and accepted as an abstract for a meeting later on this summer. We were curious as to why our method worked so well since we have not been successful in past years amplifying products from FFPE specimens, and found that the deparaffinization kit claims that it removes the formaldehyde crosslinks, which are a major reason for failed PCR. We will hopefully be able to test our protocol on additional specimens as they become available.

KEY RESEARCH ACCOMPLISHMENTS:

- A fourth manuscript is in preparation describing molecular diagnosis of fungal infections in three trauma cases from US servicemen. This manuscript is in collaboration with the infectious disease group at Brooke Army Medical Center in San Antonio (now San Antonio Military Medical Center).

- We have made substantial progress on one of our more difficult tasks, isolation of DNA from tissue and formalin fixed paraffin embedded specimens and should have an SOP for these preps.

- We have had very preliminary discussions with a startup company founded on LATE-PCR technology regarding development of the assay using a newer technology based on LATE-PCR, and may establish a collaboration in the future.

REPORTABLE OUTCOMES:

1) We have three publications in press, which have been supported by this grant (see appendix).

2) An abstract has been submitted and accepted for the Military Health System Research Symposium (MHSRS) meeting in August this year.

CONCLUSION: Throughout the first year of the project, each of the tasks that were proposed to be worked on in the first 12 months is on schedule. We have established multiple productive collaborations with military clinicians locally at BAMC (via Dr. Clint Murray) and also at Walter Reed

Medical Center in Maryland (Dr. Anuradha Ganesan). Dr. Ganesan is in the process of banking clinical specimens for the latter part of the study.

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SUPPORTING DATA: N/A

Molecular Identification of Human Fungal Pathogens

Award# 12229047

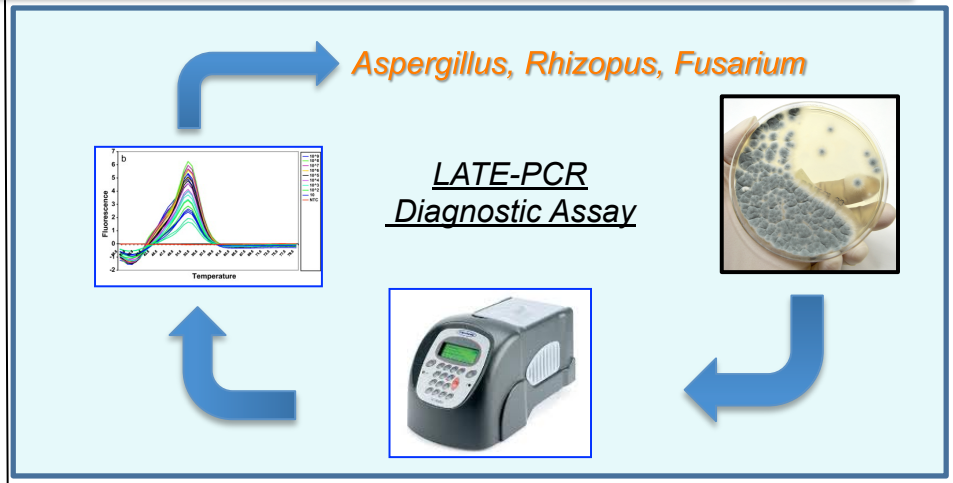
PI: Brian L. Wickes **Org:** The University of Texas Health Science Center at San Antonio

Study/Product Aim(s)

- Selection of targets suitable for a multiplex LATE-PCR reaction
- Design primers and probes for each target, to work in a single reaction
- Pilot test assay on live culture and tissue specimens

Approach

We will use our sequencing data from the previous DOD award period to develop a LATE-PCR-based assay targeted to specific fungal infections of trauma patients. We will first identify the molecular targets using our database, design and test the LATE-PCR reaction, and finally, pilot test our assay on infected tissues from trauma patients with proven fungal infections.



Accomplishments: Target design has been done. Primers design almost complete, probe design almost complete. Preliminary data suggests tissue extraction protocol is effective. Two book chapters in press, one manuscript in press.

Timeline and Cost

Activities FY	13	14
Selection of LATE-PCR targets		
Design primers and probes for targets		
Test assay on culture and tissues		

Updated: (May 29, 2014)

Goals/Milestones

CY13 Goal – SELECTION OF LATE-PCR TARGETS

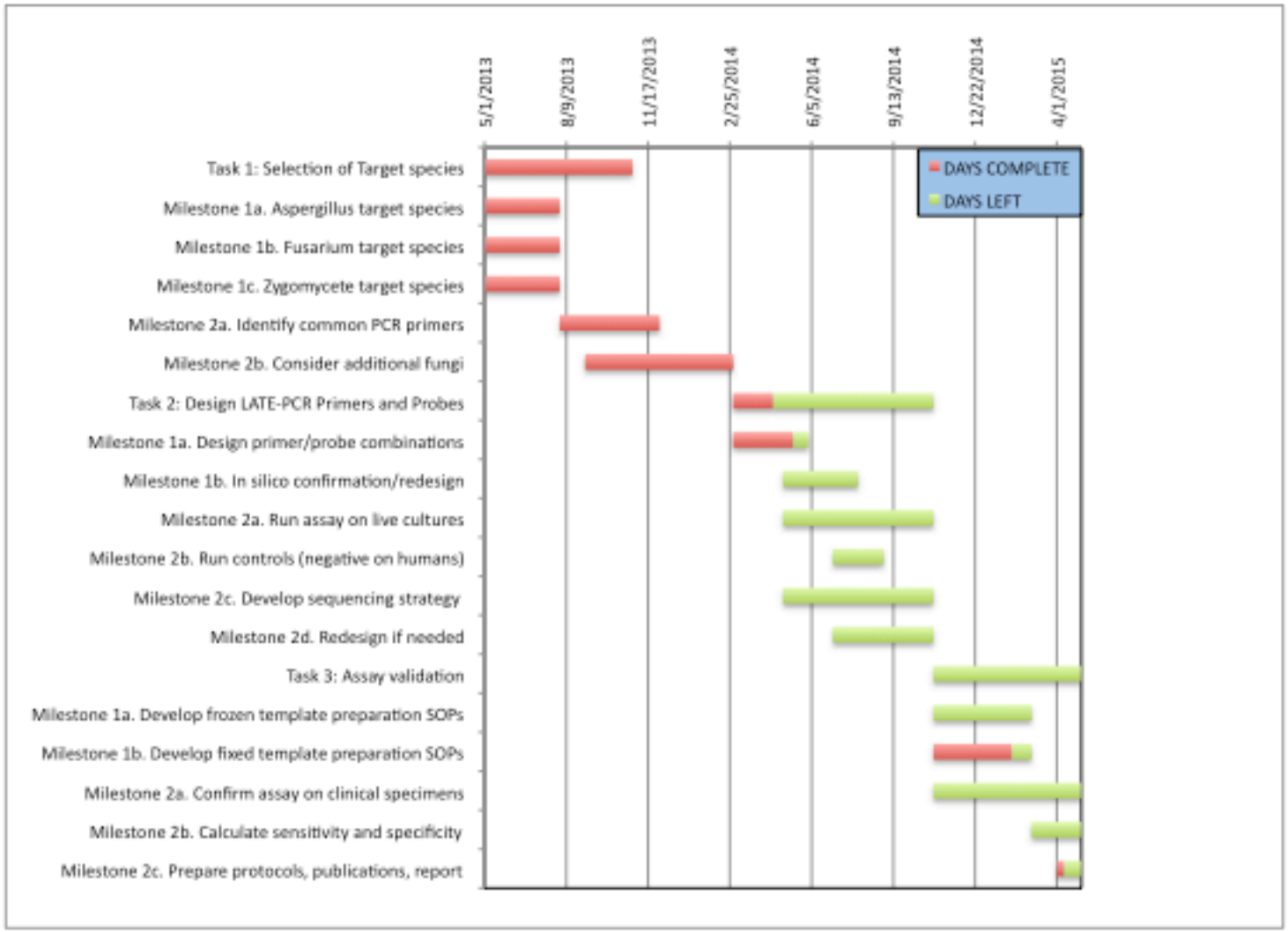
- ☑ Develop priority list of mycotic agents
- ☑ Identify molecular targets
- ☑ Design target primers and probes

CY14 Goals – VALIDATE ASSAY

- ☐ Develop protocols and SOP
- ☐ Validate assay on pure cultures
- ☐ Validate assay on tissue specimens

Comments/Challenges/Issues/Concerns

- If timelines change, comment here.
- If off by more than one quarter in spending, comment here.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Task 1: Selection of Target species
Milestone 1a. Aspergillus target species
Milestone 1b. Fusarium target species
Milestone 1c. Zygomycete target species
Milestone 2a. Identify common PCR primers
Milestone 2b. Consider additional fungi
Task 2: Design LATE-PCR Primers and Probes
Milestone 1a. Design primer/probe combinations
Milestone 1b. In silico confirmation/redesign
Milestone 2a. Run assay on live cultures
Milestone 2b. Run controls (negative on humans)
Milestone 2c. Develop sequencing strategy
Milestone 2d. Redesign if needed
Task 3: Assay validation
Milestone 1a. Develop frozen template preparation SOPs
Milestone 1b. Develop fixed template preparation SOPs
Milestone 2a. Confirm assay on clinical specimens
Milestone 2b. Calculate sensitivity and specificity
Milestone 2c. Prepare protocols, publications, report

Fungal Diagnostics

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Early diagnosis of fungal infection is critical to effective treatment. There are many impediments to diagnosis such as a diminishing number of clinical mycologists, cost, time to result, and requirements for sensitivity and specificity. In addition, fungal diagnostics must meet the contrasting needs presented by the increasing diversity of fungi found in association with the use of immunosuppressive agents in countries with high levels of medical care and the need for diagnostics in resource-limited countries where large numbers of opportunistic infections occur in patients with AIDS. Traditional approaches to diagnosis include direct microscopic examination of clinical samples, histopathology, culture, and serology. Emerging technologies include molecular diagnostics and antigen detection in clinical samples. Innovative new technologies that use molecular and immunoassay platforms have the potential to meet the needs of both resource-rich and resource-limited clinical environments.

There has been an enormous increase in the frequency and severity of fungal infection in recent years. This increase has been driven in a large part by two factors. First, the global AIDS epidemic has fostered the emergence of life-threatening infections by the opportunistic fungi *Cryptococcus neoformans* and *Pneumocystis jiroveci* and by regional endemic fungi such as *Histoplasma capsulatum* and *Penicillium marneffei*. These infections occur most often in resource-limited countries in Africa, South America, and Southeast Asia. Second, advances in medical care and treatment have led to increases in the number of opportunistic infections in patients who are immunocompromised by way of treatment with immunosuppressive drugs or chemotherapy, or who are infected in the course of extended critical care. These infections occur most often in the setting of advanced medical care. The result is patients with infections that are difficult to diagnose and difficult to treat.

Diagnosis of invasive fungal disease (IFD) is challenging because current diagnostic methods lack sensitivity and specificity, or take too long to yield a result to be clinically useful. Such limitations have consequences; delayed diagnosis leads to delayed treatment. Speed to diagnosis is a key risk factor in patient outcomes (Barnes 2008). Diagnosis of fungal infection is further complicated by problematic developments in the field of medical mycology. First and foremost is the loss of senior mycology experts in the field who were trained in classical

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mycology, which has created crises-level problems in clinical mycology (Steinbach et al. 2003). This problem has been compounded over the last 30 years as the spectrum of fungi causing infections has exploded owing to AIDS and the use of highly immunosuppressive agents for treatment of a variety of diseases. These patients are susceptible to infections from fungi rarely seen, or never reported as a human pathogen, which can cause identification problems for even the most experienced mycologists. Whereas mycologists in the past needed to be able to identify ~50 commonly encountered fungi, and ~300 total fungi that were pathogenic for humans, the number of potential fungal pathogens is likely many times what is described in textbooks, and will continue to grow as the severely immunosuppressed patient population continues to grow (Ajello and Hay 1998; Collier et al. 1998; Andrew et al. 2009).

Diagnosis of fungal infection has relied primarily on methods such as direct microscopic examination of clinical samples, histopathology, and culture. Such approaches are dependent on personnel with relatively high levels of specific mycology training. The growth in the number of fungi that clinical mycologists must identify has forced investigators to develop and apply new methods for fungal identification that go beyond classical phenotypic methods. As a consequence, there is an increased emphasis on the use of molecular methods and antigen detection as surrogates for culture in diagnosis of fungal diseases.

CULTURE, DIRECT MICROSCOPY, AND HISTOPATHOLOGY

Culture, direct microscopy, and histopathology have been the foundation for diagnosis of fungal infection for many decades. Microscopy, histopathology, and use of fungal-specific stains play important roles in diagnosis of infection by *C. neoformans*, *P. jirovecci*, *Candida* spp., *Aspergillus* spp., *H. capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Sporothrix schenckii*, *Paracoccidioides brasiliensis,* and the Mucorales. Sensitivity of microscopy for diagnosis of fungal infection varies with the individual agent, the source and quality of the specimen, and the skills and experience of the laboratorian. Finally, diagnosis of invasive fungal infection by direct microscopy and histopathology may require the use of biopsies of deep tissues, which poses a risk to those patients who are most susceptible to invasive disease.

Culture from a clinical sample is the gold standard for diagnosis of fungal infection. Culture has the advantage of yielding the specific etiological agent if positive. Moreover, culture allows for susceptibility testing. However, use of culture for diagnosis of IFD has significant limitations. Culture may take many days to a result with several of the filamentous fungi. In the case of disseminated candidiasis, blood culture (1) may miss ≥50% of patients with documented disease (Fraser et al. 1992; Ostrosky-Zeichner and Pappas 2006; Ostrosky-Zeichner 2012), (2) may only become positive late in infection (Ellepola and Morrison 2005), and (3) typically takes 24–72 h for identification of *Candida* in a clinical sample—too long for early treatment. Positive blood culture is rare in invasive aspergillosis and is most often owing to environmental contamination (Kontoyiannis et al. 2000). Recovery of *H. capsulatum* from sputum of patients with acute pulmonary histoplasmosis ranges from 10% to 15%; however, in cavitary histoplasmosis, sputum cultures are positive in up to 60% of patients (Deepe 2010). In patients with pulmonary blastomycosis, sputum culture or culture of specimens obtained by bronchoscopy has a high yield (86% per patient for sputum culture and 92% for broncoscopy) (Chapman and Sullivan 2010). Culture of *Coccidioides* spp. is complicated by the biosafety hazard associated with culture of the mycelial form. Finally, identification of less common fungi that may cause opportunistic infections requires a high level of expertise on the part of laboratory personnel.

SEROLOGY

Serologic tests for patient antibodies have been useful for non-culture-based diagnosis of fungal infection since the middle of the last century. Serology is of greatest value in diagnosis of

endemic mycoses. Available technologies include immunodiffusion (ID), complement fixation (CF) and enzyme immunoassay (EIA).

CF and immunodiffusion are the most common serologic tests for diagnosis of histoplasmosis. The ID test detects precipitating antibodies to *Histoplasma* H and M antigens. Serologic testing for histoplasmosis is most useful if an increase in CF titer is observed between acute and convalescent sera in acute histoplasmosis (Deepe 2010). High titers may be observed with chronic pulmonary or disseminated histoplasmosis (Lindsley et al. 2006).

Serological testing plays an important role in diagnosis of *coccidioidomycosis*, particularly in patients who may not be able to produce a sputum sample, for example, primary infection, or when samples are often negative, for example, coccidioidal meningitis (Galgiani 2010). Depending on the antigen used in the test, the qualitative ID test will determine the presence of coccidioidal IgM with a result that is similar to a tube precipitin test (IDTP) or coccidioidal IgG that detects antibody recognized by the CF test (IDCF). Detection of IgM is useful in diagnosis of acute primary coccidioidomycosis in which the sensitivity may be >80% (Saubolle et al. 2007). CF detects IgG antibodies. IgG antibodies are produced during the convalescent phase of disease or during chronic infection. CF is more sensitive than IDCF and provides quantitative results. A commercially available EIA can be used to detect IgM or IgG antibodies.

There are many advantages to the use of serology for diagnosis of invasive fungal infection. First, results may be positive when culture results are negative or samples are difficult to obtain. Second, if positive, serological results may reduce the need for culture of potentially hazardous fungi, for example, *Coccidioides* spp. Finally, serology is a minimally invasive sample, which lowers barriers to testing. Disadvantages of serology include sometimes low levels of sensitivity and specificity. A negative serologic test should not exclude the presence of fungal infection. Some tests, particularly CF, are time consuming and require trained personnel. Immunocompromised patients may show a reduced antibody response, which would dramatically reduce the value of serologic assays (Tobón et al. 2005). Interpretation of serological results may be confounded by the inability of serology that measures IgG to distinguish between current or previous infection. False positives may occur with some tests in the setting of other endemic fungal infections. Finally, sensitivity is dependent on the type of disease and the timing of testing relative to the disease process, for example, early versus late.

MOLECULAR DIAGNOSTICS

In the field of clinical mycology, no area is advancing faster than the application of modern molecular tools for the identification of fungi. The advancement of this area has been driven in large part by the rapid accumulation of protein and DNA sequence data, which continues unabated and accelerates with each new advance in technology, and the growing need to identify a broader range of fungi. Importantly, molecular methods for fungal diagnosis and identification directly address the declining numbers of clinical mycologists because they are not dependent on classical phenotyping methods. Similarly, molecular methods have the power to identify the increasing numbers of fungi found to produce disease in humans and animals.

Non-Culture-Based Molecular Diagnostic Methods

Molecular identification methods form a subset of diagnostic methods that do not necessarily need live fungal cells for success. Molecular methods for fungal identification generally work best when pure cultures are available. However, because polymerase chain reaction (PCR) plays **Q2** a role in many molecular identification methods, molecular identification can work in the absence of live cells if template nucleic acid is available in patient specimens, including fixed tissue.

PCR is a central component for many molecular methods, either as the main diagnostic strategy or as one of the preliminary steps in the diagnostic assay. Consequently, diagnostic PCR encompasses a number of different approaches.

The simplest consists of conventional PCR in which species-specific primers that have been designed based on existing sequence or data, are used to amplify fungal DNA from clinical specimens. The readout generally consists of the presence or absence of a band, with the size of the band often being a secondary factor in identification. This type of PCR is not FDA approved and although simple and inexpensive, can be subject to wide intra- and interlaboratory variation. There also must be some prior suspicion about the identity of the isolate, which the PCR reaction will confirm. Nonetheless, it is still used in research laboratories that occasionally may need to perform diagnostic studies on select organisms and can be suitable for minimally equipped laboratories if there is a stable power supply to run the thermocycler.

Further discriminatory power can be added to conventional PCR techniques by using restriction enzymes to digest the PCR products, which are resolved on a gel and visualized as the final step in the assay. The presence or absence of a fragment and their various sizes can then be used to make an identification. This method was used extensively in early fungal taxonomy (de Hoog et al. 2000). Unfortunately, owing to the great variability of PCR and the confirmatory nature of the technology (results are positive or negative, depending on the suspected organism), it is not widely used in the clinical laboratory. Importantly, conventional PCR serves as a confirmatory assay in which a positive reaction simply confirms the identity of the suspected fungus. The confirmatory nature of the assay is derived from the need to design primers that anneal to specific target sequences previously identified from a known organism. However, degenerate primers can expand this identification to genus or higher taxonomic levels, and if the product is to be sequenced, special strategies are used in primer design and target selection to greatly expand the number of fungi that can be amplified.

In contrast to conventional PCR, which requires gel electrophoresis of ethidium bromide stained PCR products as the final readout, real-time PCR uses fluorescent dyes to enhance specificity through either a nonspecific DNA-binding dye, that is, SYBR green, or a specific fluorescently labeled probe directed to a target sequence lying within the amplicon. There are various chemistries for probe labeling and fluorescent detection (i.e., Taqman, Molecular Beacons, etc.), as well as numerous thermocycler platforms (i.e., LightCycler, GeneAmp, etc.), which, depending on the combination, can have various levels of throughput. The application of fluorescent probes to PCR has made this technology suitable for the clinical laboratory and even has nanolevel applications that enable the identification of multiple organisms using different matrices. **Q3**

A number of assays have been FDA approved for nonfungal identification (i.e., influenza, methicillin resistant *Staphylococcus aureus*), whereas only a few have been approved for fungal identification, such as the FilmArray Blood Culture Identification (BioFire Diagnostics, Inc.), which is a PCR-based reaction performed on positive blood cultures and detects mainly *Candida* spp. Because PCR-based assays include an amplification step of a specific nucleic acid target, they can be performed on specimens that are potentially contaminated with human tissue or fluids, which is a tremendous advantage of PCR because a time-consuming outgrowth period is not needed and the assay can often be completed in a few hours. Furthermore, PCR can be performed on fixed tissue with some success, although these templates come with special challenges (Dannaoui et al. 2010). Like conventional PCR, real-time PCR generally is confirmatory in that a suspicion of the organism identity is established before performing the PCR reaction. Additionally, although multiplexable, a problem with probe-based diagnostic assays is that each assay must contain all probes for the organisms it can detect, which can entail substantial reagent costs, even when performed on a nanoscale.

Culture-Based Molecular Diagnostic Methods

Establishing a pure culture of a suspected microbial sample isolated from a clinical specimen has always been the gold standard of diagnostic

microbiology, regardless of the downstream diagnostic assay that is applied for the ultimate identity of the organism. Although in many instances it is not possible to obtain a pure, viable culture, virtually all diagnostic assays work best if the assay initiates from a pure culture. Pure cultures ensure there is enough material to perform the assay, enable repeat assays in the case of failure, allow for sending the isolate to alternate sites where the assay can be performed, and/or allow additional unrelated assays to be performed if confirmation or a more discriminatory strategy is needed. Culture-based methods historically have been phenotype driven, in which yeasts were identified biochemically and molds were identified based on morphological features. These methods are still important, frontline diagnostic methods. However, with the application of molecular biology to fungal taxonomy and phylogeny, it has become clear that molecular biology needs to be part of diagnostic mycology. As a result, the major molecular taxonomic tool, ribosomal sequencing, is now the major molecular tool for fungal identification.

Sequencing of fungal ribosomal targets is an attractive diagnostic method for a number of reasons. First, fungal ribosomes and most eukaryotic ribosomal genes are multicopy in nature, which increases detection sensitivity during PCR amplification because there are more target sequences. Second, the organization of these loci in fungi places multiple conserved ribosomal (18s, 5.8s, and 28s) subunit genes in close proximity, which offers conserved PCR primer sites that are positioned such that multiple target sites are close enough to yield PCR products. In fact, the conserved nature of the subunits, and their primer annealing sites, makes PCR and sequence identification possible, enabling virtually any unknown fungus to be amplified with universal primers targeted to these regions (White et al. 1990; Kurtzman and Robnett 1997). Third, the overall organization of this region confers variability owing to the fact that variable regions separate the key ribosomal units. These regions, called the internal transcribed spacer (ITS) sequences, consist of two regions (ITS1 and ITS2) that are not part of the fungal ribosome and are spliced out after transcription (Fig. 1). Their presence confers the sequence variability that makes rDNA sequencing the most powerful nucleic acid–based diagnostic method available. In addition to the variable ITS1 and ITS2 regions, a third variable region exists within the large 28s ribosomal subunit called the D1/D2 region. All three regions are informative owing to their variable nature, which can yield genus-specific and species-specific identifications. Finally,

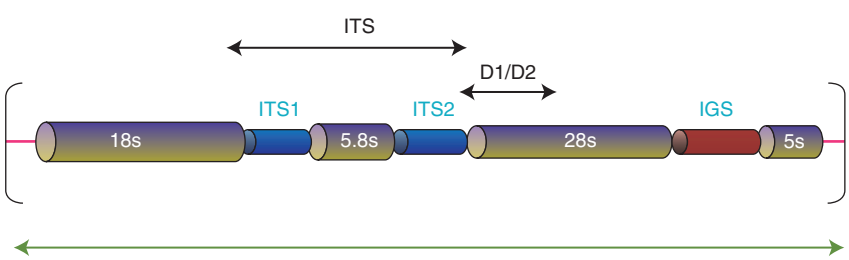

Figure 1. Ribosomal subunit organization in eukaryotes with variable regions. Eukaryotic ribosomal subunit genes are typically organized in repeats of the 18s rDNA (small subunit), ITS1 (internal transcribed spacer region 1), 5.8s rDNA, ITS2 (internal transcribed spacer region 2), and the 28s rDNA (large subunit). Conserved priming sites exist at the end of the 28s subunit and the beginning of the 28s subunit, and at the end of the D1/D2 region within the 28s subunit. Additional sites exist within the 5.8s subunit and throughout the large and small ribosomal subunits. The variable regions, which can provide information that can discriminate to the species level, depending on genus, include ITS1, ITS2, and D1/D2. The ITS region can be covered in a single PCR reaction and by double-stranded sequencing, and is roughly 450–750 bp in length, depending on species. The D1/D2 region is in a similar size range.

sequence data generated from an unknown fungus can be used to search the public databases, such as GenBank, using the web-based BLASTn algorithm.

Database searches must be performed with extreme caution owing to the public nature of the database and high frequency of erroneous deposits (Bidartondo 2008). Nevertheless, there are hundreds of thousands of fungal sequences deposited within GenBank that can serve as potential identification data. It is the wide array of different fungal species that makes GenBank so powerful. It is unlikely that any fungal identification platform will be as broad in terms of the number of potential species that can be identified using DNA sequences because of the public nature of GenBank, length of time sequences have been deposited into the database, and widespread use of sequencing as a basic molecular technique. In fact, most journals that publish papers that include sequence information require a GenBank deposit of the data. Importantly, global access to GenBank ensures that fungal sequences from all over the world are regularly deposited, which serves to increase the diversity of information in the database. Unfortunately, the unedited nature of GenBank has always been problematic for fungal identification, which has resulted in the development of both commercial and publicly curated, closed databases.

Although most of the taxonomically significant sequences are ribosomal in nature, for some fungi, there is not enough specificity in the ribosomal loci to discriminate different species. In this case, alternate sites based on conserved structural genes have been developed and in many cases are standard depending on the presumed identity (Table 1) (Petti et al. 2008). However, to generate these sequences, specific primers must be used, which requires some information about the unknown fungus to guide primer selection. Some genes such as tubulin have primers that amplify across families (Glass and Donaldson 1995).

A requirement for sequence-based diagnostics is the need for bioinformatics skills to manipulate sequences and ultimately to search databases and interpret the results. This requirement has led to the development of alternate DNA-based identification technologies. One of these technologies (Luminex xMAP) uses color-coded microspheres that are specific for each analyte in a sample. In the case of fungal identification, this platform uses PCR of an unknown sample that can then be detected in a hybridization assay by binding to its corresponding bead in the assay. Detection and identification using this methodology is similar to the technology used in flow cytometry. Each assay is capable of detecting up to 100 different species in a single multiplex reaction (Preuner and Lion 2013). The advantage of this platform is that there are no downstream manipulations of data after the assay is complete. The multiplex capability also greatly exceeds a single PCR reaction; however, the true identity of the organism must be one of the 100 components of the multiplex.

Proteomics Profiling/Fingerprinting

The most popular and fastest growing non–nucleic acid sequence based molecular diagnostic assay for fungi is MALDI-TOF (matrix-assisted laser desorption/ionization time of flight). The technique generates species-specific spectra that provide a unique signature characteristic of the species. MALDI-TOF instrumentation consists of an ion source that transfers sample molecules into a gas phase, a mass analyzer that resolves ions based on mass-to-charge ratio, and a component that detects the ions (Croxatto et al. 2012). Samples are mixed with a matrix of small acidic molecules that crystallizes the specimen and facilitates ionization because the matrix absorbs energy in the range of the laser used for sample excitation. The TOF component consists of a tube that the excited ions travel through, with the transit time (time of flight) of individual ions providing the method for identification. The generated spectra are screened against a library of reference spectra, which correspond to individual species. The technology has already been commercialized for microbial identification, with instruments being available from Bruker Daltronics (MALDI Biotyper), Shimazu (AXIMA@SARAMIS),

Table 1. Alternate non-rDNA loci used for molecular identification

Locus	Genera/order	Reference
β-Tubulin	*Aspergillus*, *Penicillium*, *Phoma*, *Pseudallescheria*, *Chaetomium*, *Phaeoacremonium*, *Sporothrix*, *Paecilomyces*	Glass and Donaldson 1995
Preribosomal-processing protein	*Aspergillus*, *Penicillium*	Houbraken and Samson 2011
Minichromosome maintenance protein	*Aspergillus*	Sugui et al. 2012
Translation elongation factor 1α	*Fusarium*, Mucorales, *Beauveria*, *Cordyceps*, *Trichoderma*, *Alternaria*, *Cladosporium*, *Phomopsis*	O'Donnell et al. 1998, 2001; Abe et al. 2010
Cytochrome oxidase 1	*Penicillium*	Lasker 2006
Calmodulin	*Pseudallescheria*, *Sporothrix*, *Aspergillus*	O'Donnell et al. 2000; Hong et al. 2006; Marimon et al. 2006
Glyceraldehyde-3-phosphate dehydrogenase	*Cochliobolus*, *Curvularia*, *Bipolaris*	Berbee et al. 1999
Endopolygalacturonase	*Alternaria*	Andrew et al. 2009
RNA polymerase II	*Schizophyllum*, *Alternaria*, *Penicillium*, *Aspergillus*	Liu et al. 1999; Peterson 2008; Samson et al. 2011; Yilmaz et al. 2012
Actin	*Rhizopus*, *Stachybotrys*, *Trichophyton*, *Phaeoacremonium*	Carbone and Kohn 1999; Gao and Takashima 2004
Intergenic sequence	*Trichosporon*	Sugita et al. 2002

Andromas (Andromas), and bioMérieux (Vitek MS) (Bader 2013).

The strength of MALDI-TOF technology lies in the rapid sample analysis (minutes) and the absence of any downstream data manipulation. For many fungi, particularly many yeasts, sample prep is minimal. For other fungi, a rapid extraction with a solvent is all that is needed. These factors—the lack of downstream steps and simple sample preparation—combined with the accuracy and speed of this system make MALDI-TOF one of the most intriguing diagnostic options for fungal identification. Importantly, just as for sequencing, no prior suspicion of the true identity of the isolate is needed. If there is a reference spectrum in the library, there is a high probability that a correct identification can be made (Posteraro et al. 2013). Weaknesses of this system include the need for an existing spectral library to compare generated spectra to, and potential variability in results of unknown fungi if they are not grown under conditions similar to reference spectra in the library. Libraries are proprietary and instrument specific, although most are modifiable by users. There are capitalization costs for the instrumentation, and user skills need to be somewhat advanced. Portability is also not an option with this instrumentation.

Challenges to Molecular-Based Diagnostic Mycology

There remain a number of challenges preventing full implementation of a molecular-based platform as the main identification method in clinical microbiology laboratories. On the mycology side, the spectrum of fungi that can cause disease continues to grow and likely will be almost infinite as patient populations, depending on illness or treatment, can be so profoundly immunosuppressed that species never before seen as pathogenic for humans regularly appear in the clinical microbiology laboratory. Identifying these fungi using classical phenotypic methods is extremely difficult as laboratory staff

generally do not have the necessary training or experience to arrive at an identification based on morphology for rare fungi. Biochemical identification is generally limited to commercial platforms that have panels of standard compounds, which are directed toward identifying common fungi found in a coded database depending on metabolism pattern of the panel. Furthermore, mycology is unique in that there is a special nomenclature system that takes into consideration whether or not a given isolate has a sexual state or not. Unfortunately, only the most experienced mycologists can navigate this system, which has become so complex that efforts are under way to reform it so that a simpler more stable naming system can be used (Hibbett and Taylor 2013). Beginning in 2013, consistent with the Amsterdam Declaration on Fungal Nomenclature, a unified effort will be made to insure that the one-fungus–one-name rule is implemented so that fungal nomenclature can be simplified (Hawksworth et al. 2011).

On the technical side, one of the simplest, yet longest running challenges impeding molecular diagnostics is a universal method for preparing sample templates. For sequence-based assays, fungi vary depending on their morphology in their resistance to cell lysis and ease of release of nucleic acid. There are countless methods that have been successful, ranging from enzymatic to chemical to physical; however, a "one size fits all" method that could be commercialized has proven elusive. This problem also hinders development of strategies that could meet FDA approval because lack of consistency and varying requirements for technical input are problematic for certified laboratories. Additionally, the sheer number of fungi that a microbiology laboratory must be able to identify can preclude the use of assays, such as PCR, that can only identify one or a few fungi in the given assay. More open-ended assays, which theoretically can identify any species within a given platform's reference libraries or databases, are more realistic because they could potentially replace all culture-based assays in the clinical laboratory, and can even be used across kingdoms. However, these assays tend to require expensive, stationary equipment that requires specialized skills to operate.

ANTIGEN DETECTION

Fungal polysaccharides or proteins may be shed into body fluids during the course of infection. If an antibody can be raised against such a shed antigen, an immunoassay can be constructed for antigen detection.

Cryptococcosis

Diagnosis of cryptococcal meningitis was the first application of antigen detection for diagnosis of fungal infection that received widespread clinical use (Bloomfield et al. 1963). Antibodies were raised in rabbits against whole cryptococcal cells and passively coated onto latex beads. Termed latex agglutination, the assay detected glucuronoxylomannan (GXM), the major capsular polysaccharide of *C. neoformans*. GXM is shed in large amounts into blood and cerebrospinal fluid (CSF) during the course of cryptococcal meningitis.

GXM occurs in four major serotypes: A, B, C, and D and a hybrid serotype AD. Before the use of molecular methods for classification of cryptococcal species, serotypes A, D, and A/D were *C. neoformans*; serotypes B and C were *C. gattii* (Bennett et al. 1977; Kwon-Chung and Bennett 1984b). With further study, *C. neoformans* var. *grubii* was identified to correspond to serotype A. *C. neoformans* var. *neoformans* corresponds to serotype D.

There is considerable variability in the geographic distribution of the cryptococcal species and their corresponding serotypes. Serotype A has a global distribution, and initial studies of cryptococcosis in patients with AIDS found a predominance of serotype A isolates, suggesting that serotype A was selectively infecting AIDS patients. However, there are several recent reports of serotype C in AIDS patients in sub-Saharan Africa where the frequency of serotype C has been reported to be as high as 14% (Karstaedt et al. 2002; Litvintseva et al. 2005; Thakur et al. 2009). *C. neoformans* var. *neoformans* (serotype D) has a global distribution, but clinical

cases are concentrated in Europe (Kwon-Chung and Bennett 1984a; Dromer et al. 1996). Finally, *C. gattii* has gained recent prominence as the cause of an ongoing outbreak that began in Vancouver Island, British Columbia. Although there has not been an extensive serological characterization of isolates from the outbreak, molecular typing has found that almost all isolates are genetic variants of the VGII class on the basis of PCR fingerprinting; isolates of VGII produce capsules of serotype B (Litvintseva et al. 2011). Geographic variability in occurrence of cryptococcosis of different serotypes has consequences for diagnostic testing that targets cryptococcal antigen; assays for antigen must be able to detect GXM of all major serotypes.

A significant advance in testing for cryptococcal antigen was the development of an assay in lateral flow immunoassay (dipstick) format. Termed CrAg LFA, the assay is constructed from a cocktail of monoclonal antibodies that were formulated to be reactive with all GXM serotypes (Gates-Hollingsworth and Kozel 2013). The CrAg LFA is particularly well suited for use in resource-limited settings. The LFA requires no power or clean water, is inexpensive, requires no refrigeration, and can be performed by personnel with limited training. The LFA works well with serum or a drop of blood and provides a result in ~10 min. As a consequence, patients can be treated at the time of an initial visit to clinic. The ease of use of the CrAg LFA also makes the test valuable for use in settings with advanced medical care.

Recent studies have found that CrAg testing can be used for prospective testing in asymptomatic patients at high risk for cryptococcosis. CrAg is present in serum for weeks to months before the onset of symptoms of cryptococcosis (Jarvis et al. 2009). As a consequence, a targeted screening program could identify subclinical infection in patients at greatest risk for cryptococcal meningitis. Specifically, HIV-infected patients can be screened for CrAg at the time of initial diagnosis of HIV/AIDS before starting antiretroviral therapy (World Health Organization Cryptococcal Working Group 2011). If a patient tests positive in this prospective screen, the patient can be preemptively treated to prevent infection from progressing to meningitis.

Galactomannan

In 1978, Lehmann and Reiss identified an antigen in serum from immunosuppressed rabbits infected with *Aspergillus fumigatus* (Lehmann and Reiss 1978). The investigators later found that the same antigen is released from growing hyphae (Reiss and Lehmann 1979). This antigen is galactomannan (GM), a polysaccharide present in the cell wall of most *Aspergillus* spp. A test for serum GM is now in widespread use for diagnosis of invasive aspergillosis (Platelia Aspergillus, Bio-Rad Laboratories). The test is an enzyme immunoassay that uses a rat monoclonal antibody that recognizes β (1 → 5)-linked galactofuranose (Stynen et al. 1992, 1995). Multiple studies have found the GM enzyme immunoassay (EIA) to be useful in diagnosis of invasive aspergillosis in neutropenic patients with cancer and recipients of stem cell transplants (Machetti et al. 1998; Maertens et al. 1999, 2001; Sulahian et al. 2001). **Q4**

There are limitations to the GM immunoassay for diagnosis of invasive aspergillosis. First, there is considerable variability in reports of sensitivity and specificity (Machetti et al. 1998; Maertens et al. 1999, 2001; Pinel et al. 2003). Second, false positive reactions may occur owing to a variety of factors, including administration of β lactam antibiotics (Pinel et al. 2003) or infusion of gluconate-containing Plasma-Lyte (Petraitiene et al. 2011). Third, there is considerable cross-reactivity with other fungi producing disseminated infection (Huang et al. 2007; Wheat et al. 2007; Xavier et al. 2009).

Detection of *H. capsulatum* polysaccharide antigen in body fluids, especially urine, has been useful in presumptive diagnosis of histoplasmosis in patients with disseminated disease (Wheat et al. 1986). The antigen is a galactomannan (Connolly et al. 2007). Antigen can be detected in urine of ~90% of patients with disseminated infection (Williams et al. 1994; Durkin et al. 1997). The sensitivity of antigen detection is greater in urine than in serum (Wheat et al. 2002). This is an antigen-capture ELISA in

which polyclonal rabbit antibodies are used in both the solid-phase capture and fluid-phase indicator modes. Galactomannan recognized by immunoassay of urine in histoplasmosis patients is cross-reactive with polysaccharide antigen produced by several endemic mycoses (*B. dermatitidis*, *P. brasiliensis, H. capsulatum* var. *duboisii*, and *P. marneffei*) (Azuma et al. 1974; Wheat et al. 1997).

Immunoassays have been recently developed for detection of galactomannan from *B. dermatitidis* and *C. immitis* in urine and other body fluids. For *B. dermatitidis* antigen detection, antigenuria was detected in 90% of patients with culture- or histopathology-proven blastomycosis (Connolly et al. 2012). Specificity was 99% in healthy subjects and patients with nonfungal disease, but cross-reactions occurred in 96% of patients with histoplasmosis. For *C. immitis* antigen detection, antigenuria was detected in 71% of patients with more severe forms of coccidioidomycosis (Durkin et al. 2008). Additional studies are needed to assess the usefulness of galactomannan testing in other clinical forms of coccidioidomycosis.

Pan-Fungal Detection of β-Glucan

(1 → 3)-β-D-glucan (BG) is a polysaccharide component of most fungal cell walls. The polysaccharide may be released into blood in the course of IFD, including infection by species of *Aspergillus*, *Candida*, *Fusarium*, *Trichosporin*, *Saccharomyces*, *Acremonium*, and *P. jiroveci*. BG does not appear in blood from patients with infection by *Cryptococcus* spp. or the Mucorales. The assay for BG is not an immunoassay. Rather, assay for BG is based on the ability of the polysaccharide to activate factor G of the horseshoe crab coagulation cascade. With use of a chromogenic substrate, the test can detect BG levels as low as 1 pg/mL (Obayashi et al. 1995).

Assay for BG has value as a screen for presumptive diagnosis of invasive fungal infection (IFI). Early diagnosis allows for earlier initiation of antifungal therapy. Clinical studies have found BG testing to have a high sensitivity for diagnosis of IFI. The test has a strong negative predictive value, allowing the test to be used to exclude IFI. There are a number of limitations in the use of BG assay for diagnosis of IFI. First, BG is ubiquitous in the environment, which may produce false positive reactions. All testing materials must be glucan-free. Second, BG testing is typically performed at reference laboratories, which reduces time to result and discourages routine, potentially prospective, testing by clinicians. Finally, BG testing cannot be used to detect mucormycosis or cryptococcosis.

Strengths and Weaknesses of Antigen Testing

A particular advantage of testing for antigen is the possibility that antigen can be shed from a local site of infection to a body fluid such as blood or urine. As a consequence, it is possible to avoid highly invasive sample collection. Circulating or urinary antigen functions as a surrogate for the actual presence of the microbe. Second, it is possible to use antigen detection platforms that can be inexpensive, rapid, and capable of use by personnel with limited training. This is the case with the CrAg LFA for diagnosis of cryptococcosis. Third, by judicious selection of antibodies, it is possible to produce a test with a broad or a very limited specificity, depending on the clinical needs for testing. A major limitation for antigen testing is the need to identify antigen surrogates for infection. CrAg is a well-characterized surrogate for infection; however, identification of similar surrogates for other infections is needed.

CONCLUDING REMARKS

Desirable properties of a diagnostic test are listed in Table 2. It is likely that several diagnostic platforms will be needed to meet the diverse requirements of different fungal infections and the resources available for testing. The ideal test would detect infection early in the course of disease, perhaps before the advent of symptoms. Early diagnosis would enable administration of antifungals at a time when treatment is most likely to be effective. For example, there is a 20% increase in mortality of invasive candidiasis if therapy is delayed by >12 h (Morrell et al.

Table 2. Properties of ideal next-generation fungal diagnostics

Detects infection early in course of disease
Strong negative predictive value
Short time to result; ideally near point of care
Low cost; inexpensive equipment/instrumentation
Use of noninvasive sample
Suitable for use by personnel with limited training
Minimal number of steps
Uncomplicated interpretation; no downstream analysis required

2005). An example of a diagnostic test that can identify subacute infection is detection of cryptococcal polysaccharide in sera of patients who enter antiretroviral therapy who do not have symptoms of cryptococcal meningitis (Jarvis et al. 2009). Second, a test with strong negative predictive value would be of great value in managing patients at high risk for IFD. The neutropenic patient with fever of unknown origin that is unresponsive to broad-spectrum antibiotics is often treated empirically with antifungal agents. A test with a high negative predictive value could identify patients who should not be given antifungal therapy. This would reduce the cost of patient care and reduce development of antibiotic resistance.

Many laboratory tests for diagnosis of fungal infection are typically performed at reference laboratories, for example, tests for galactomannan or BG. The time to result presents a barrier to routine use of such tests. Similarly, high cost presents barriers to routine use of advanced testing for fungal infection. A major barrier to use of many diagnostic approaches is the need for invasive procedures for sample collection, particularly for high-risk patients. The ideal test would use readily accessible samples such as blood or urine. An example is the CrAg LFA, which has a high sensitivity for diagnosis of cryptococcosis using serum. This avoids the need for the lumbar puncture, a major advantage in resource-limited settings (Jarvis et al. 2013). Similarly, testing of urine for *Histoplasma* galactomannan enables diagnosis of disseminated histoplasmosis and is useful in early diagnosis of acute pulmonary histoplasmosis and in treatment follow-up (Wheat 2006). Finally, most diagnostic tests for IFI require a high level of operator expertise. Once again, this need for a relatively advanced infrastructure reduces access and raises barriers to testing.

New generations of fungal diagnostics must reconcile the diverse needs of patients in developed countries with advanced levels of medical care and those of patients in resource-limited settings. Many of the IFIs in countries with advanced medical care are a consequence of the ability to provide long-term critical care to patients or the use of highly immunosuppressive agents. In contrast, most deaths owing to fungal infection globally occur in patients with AIDS, most often in settings with little or no infrastructure. On a global level, fungal diagnostics must be able to function in a setting of limited infrastructure.

Is it possible to reconcile the needs of resource-limited countries and those of patients in settings of advanced medical care? Diagnostic tests that meet the criteria outlined in Table 2 may be able to do so. Clearly, low-cost testing that can be performed at the point-of-patient care is critical in resource-limited countries. However, these same tests may be able to dramatically improve outcome for the critically ill patient in advanced medical settings. For example, if testing could be performed at low cost with a noninvasive sample and produce a rapid result, such testing would enable prospective monitoring of those patients at high risk for IFD. Prospective monitoring would identify infection before the occurrence of overt symptoms and enable early treatment when it is most likely to be effective.

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Queries

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Q1 Please check spelling change to "dermatitidis"

Q2 Please check definition of PCR.

Q3 Please check. SYBR meant here?

Q4 Please check definition of EIA.

Q5 Please check all information in Barnes 2008.

Fungal Diagnostics

Thomas R. Kozel and Brian Wickes

TOC Blurb: New generations of fungal diagnostics must reconcile the needs of patients in developed countries with those in resource-limited settings. Emerging technologies that use molecular and immunoassay platforms may meet these needs.

Diagnosis and Treatment of Fungal Infections, 2nd Edition

Part II: Laboratory and Radiological Diagnosis

Chapter 3. Diagnostic Molecular Biology

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ABSTRACT

Clinical mycology is being increasingly challenged by two growing problems: 1) loss of experienced medical mycologists that can identify both common and rare fungi, and 2) a rapidly expanding group of clinically significant fungi that clinicians have rarely or never seen before that are appearing with increasing frequency. Because increasingly fewer young, experienced, medical mycologists are entering clinical laboratories, the skills to identify current and future new species of fungi are rapidly disappearing. Unfortunately, patient populations are becoming increasingly immunosuppressed for longer periods of time, which is creating a perfect storm of a growing need for diagnostic skills being unmet by decreasing numbers of young mycologists entering the clinical profession. One of the ways around this issue to is develop new, robust, easy to use diagnostic strategies. The evidence is overwhelming that the field of molecular biology can be the source of these new strategies. There currently are multiple approaches that employ new equipment and new techniques that offer a way to fill the growing diagnostic vacuum that has been created in the last ten-twenty years. These approaches need to make their way into clinical microbiology laboratories as quickly as possible in order to insure that the diagnostic needs for the field of medical mycology are met today and into the future.

1. INTRODUCTION

The role of molecular biology in the clinical microbiology laboratory continues to grow at an ever-accelerating pace. Few areas of diagnostic microbiology stand to benefit more from the application of molecular diagnostics than clinical mycology. The increasing role of molecular biology in fungal diagnosis and identification is driven by two factors. First is the continually expanding spectrum of clinically significant fungi that are being recovered from patients. This list grows every year and is directly related to the growing population of immunocompromised patients at risk for the development of serious fungal infections. AIDS has been perhaps the greatest wake-up call with regard to what awaits the immunosuppressed, and organ transplantation has provided one of the strongest reminders of how desperate medical mycology is for new classes of antifungals. Second, the field of diagnostic mycology has been described as being in crisis due to the lack of experienced mycologists who can identify the increasing numbers of never before seen fungi that are recovered from patients (1). The United States in particular, and most countries in general, abandoned education and training programs in classical mycology, which traditionally produced a steady stream of microbiologists that could identify fungi beyond the most common ones routinely encountered in a clinical microbiology laboratory. Consequently, there are few young scientists being educated and trained today to replace the retiring clinical mycologists who have expertise in classical mycology that can be translated into fungal identification using traditional phenotypic (ie., morphology) methods. The lack in this skillset, combined with the increasing need for this expertise is having downstream repercussions that translate into increased morbidity, mortality, and healthcare costs. Furthermore, there are troubling trends in the frequency of fungal infections that place added pressure on clinical laboratories. For example, *Candida* infections, now the fourth most common nosocomial infection, carry an estimated increased cost of $68,311 and an extra 23 days in the hospital (2), part of the 2.6 billion dollar expenditure on systemic

fungal infections in just the United States alone (3).

Clinicians recognize that in contrast to bacterial infections, which allow multiple antibiotic treatment options, fungal infections must be treated using a very limited selection of antifungal drugs, many of which carry substantial side effects or work poorly in certain patient populations. However, time of diagnosis of systemic infection is a proven risk factor for increased morbidity and mortality (4), confirming the important role that mycology diagnostic expertise in the clinical microbiology laboratory plays in healthcare, both from a cost and patient outcome perspective. The options for strengthening this role in light of diminishing expertise is increasingly falling on technology and more specifically, to molecular biology, as potential ways to make up lost ground by providing new tools for diagnostic mycology. There remain, however, many challenges to overcome before the full power of molecular biology can be applied in the clinical laboratory.

2. CURRENT STATE OF FUNGAL DETECTION IN THE CLINICAL LABORATORY

Traditionally, fungal identification has largely been based on the subjective micro- and macroscopic examination of morphological and culture characteristics. As such, these evaluations rely on the expertise of a trained mycologist and therefore are subject to variation in identification depending on the skill and experience of the microbiologist. There are also numerous genera, species, and strains that show morphological similarities, which can also lead to fungal misidentifications (5). The ability to rapidly and correctly identify positive clinical specimens has immense potential to impact the therapeutic decisions regarding empirical antifungal therapy.

Increasing numbers of immunosuppressed patients, coupled with a widening range of recognized pathogens, and the discovery of resistance of some yeasts to antifungal drugs mean that the common practice of identification or exclusion of *Candida albicans* alone is no longer adequate (6).

The clinical microbiology lab is somewhat limited in the ability to differentiate and identify less frequently encountered yeasts. Some of the methods routinely used to identify yeasts in a clinical lab are Gram stain, germ tube production, culture (primarily onto chromogenic media or cornmeal agar to aid in species differentiation), biochemical methods including commercial platforms such as API 20 C AUX (bioMérieux, Inc., Durham, NC), use of biomarkers (Galactomannan and 1,3-ß-D-glucan (BDG), and PNA-FISH. Culture methods, lactophenol cotton blue staining, and more recently, PCR platforms, are used for the identification of moulds in the clinical microbiology lab (6, 7). Historically, the clinical microbiology laboratory has relied heavily on conventional methods for fungal identification, which is often not practical because they are labor intensive and can take days to weeks to complete, depending on the isolate. Therefore, there is a constant need for new approaches allowing for the rapid and accurate identification of pathogenic fungi.

3. CURRENT CHALLENGES IN DIAGNOSTIC MOLECULAR BIOLOGY.

Presently, there are numerous molecular-based methods for the identification of fungi that are in various stages of development, but are mainly used for research purposes. They have been slow to find their way into clinical laboratories due to a number of factors that need to be overcome before molecular diagnosis becomes the predominant or even routine application in a clinical environment. From a research perspective, many of the newer technologies have proven to be valuable and have potential for a future role in the clinical laboratory. There are, however, numerous hurdles that need to be overcome before some of the current technologies can become a major work horse in the clinical laboratory.

3.1 Instrumentation

Many molecular assays require expensive equipment platforms that bring high capitol costs, which can be in the range of $100,000 or more depending on the instrument. For fungi, some of the larger platforms include DNA sequencers for sequence analysis or mass spectrometry instrumentation, such as Matrix-Assisted Laser Desorption/Ionization (MALDI), which is used for analysis of biomolecules (DNA, protein, etc.). In addition to high initial capitol costs, not including any supportive equipment, these instruments carry annual service contracts that are typically in the range of 10% of the purchase price. They may also have additional requirements such as special electrical requirements that include back up power sources or separate electrical lines that are free of surges, large footprints, or space requirements that must insure they are not moved or agitated due to precise calibration requirements. For some instruments, continued development has brought down the size and cost of many platforms used in molecular biology, with many approaching bench top size and affordability. Thermocyclers used for real time PCR have dropped drastically in size and price over the years and can be configured to support high throughput applications and easily interfaced with a computer for complex data management and analysis. Importantly, one of the major criteria involved in the decision making process of whether or not to purchase an instrument is throughput. Most clinical laboratories are not going to have the fungal identification throughputs to justify large capitol outlays. However, for some types of analyses, such as sequencing, cross-microbe application can justify the associated expense.

3.2 Expertise

In addition to instrumentation costs, expertise to operate the equipment often imposes additional salary pressure on microbiology laboratories. There is great pressure to develop assays and

instruments that are operated by loading the specimen and retrieving data, with little or no pre-sample or post assay analysis or manipulation required. However, in many cases this simplicity is not possible. The instruments themselves can be complicated to run and most importantly, downstream analysis and manipulation of data can require expertise not found in traditional clinical microbiology or medical technology training. Furthermore, unless analysis is performed in a reference laboratory, specific expertise almost always would need to be translatable between sub disciplines since workloads may not be enough to justify investment in instrumentation or personnel dedicated to a single microbe (i.e., fungi, parasites, etc).

3.3 Throughput and turnaround time.

The importance of rapid diagnosis of an infecting fungal pathogen cannot be overstated given the significance that this risk factor plays in morbidity and mortality. For some patient types, it may be the most important criterion that affects survival. For example, Von Eiff et al., showed that starting antifungal therapy for pulmonary aspergillosis after 10 days increased mortality rates from 40% to 90% (8). Unfortunately, due to potential cost, throughput becomes an issue in molecular diagnostics because there must be enough savings per isolate, through speed of identification, accuracy, and labor-saving methodology to justify implementation of a given molecular assay. For smaller platforms, such as PCR, thermocyclers can be used on non-fungal microbes (ie., bacteria, viruses), and/or have additional applications beyond microbial diagnostics. These instruments are generally inexpensive to moderately expensive and may be within the operating budget of the clinical laboratory. Certainly larger reference laboratories are better positioned to support this equipment. However, an important factor to consider for any platform is per assay cost. Many instruments will have pre-packaged reagents dedicated to a given assay, which will be much more expensive than homemade recipes.

Furthermore, in order to meet certification requirements in a clinical laboratory, specific assays will be more expensive to run than they would be in a research laboratory. For example, a research laboratory may run sequencing reagents at 1/8, 1/16 or even 1/32 concentration, depending on laboratory expertise, while a certified laboratory may need to run full strength reactions to insure inter laboratory consistency as mandated by the appropriate accrediting agency (ie., College of American Pathologists, Clinical and Laboratory Standards Institute).

3.4 Template preparation

There is probably no greater challenge to molecular diagnostics than template preparation because this issue impacts every other component of molecular diagnostics. Fungi are unique from virtually all other medically important microbes in that lysing the cells can be problematic and varying in difficulty depending on the genus. Additionally, once lysed, fungal cellular material, such as melanin or polysaccharides, can inhibit downstream reactions and lead to erroneous or negative results unless it is removed. Because almost all fungi have a tough cell wall, extractions can require harsh, laborious, or prolonged manipulations. For any diagnostic assay requiring an amplification step, such as PCR, multiple sample manipulations that contain excess tube transfers should be avoided because each transfer introduces a potential opportunity for external DNA contamination. Unfortunately, this problem is ever present as even some commercial material used for DNA purification can be contaminated with exogenous fungal nucleic acid that can be amplified during PCR (9). Using alternative methods, such as physical breakage or enzymatic spheroplasting and lysis are suboptimal because they typically require multiple time consuming, laborious, organic extractions and precipitations that eventually yield a DNA amount much larger than what is needed for the assay (Fig. 1). Automation may offer a suitable alternative because it can be standardized using specific fungal

nucleic acid extraction programs and/or reagents, which give excellent yields that enable multiple downstream assays. However, this approach requires instrumentation that may be beyond a laboratory budget or which may not be used enough to justify the purchase.

An unfortunate characteristic regarding fungal template preparation is that templates can be from a variety of sources, not just pure culture. The advantage of having a live culture is that there is a limitless supply of starting material for template preparation because any problems can be addressed by simply subculturing and starting over. However, the absence of a pure culture can be quite common in fungal infections, which in fact, can increase the value of any molecular assay if pure cultures are optional. Instead, starting material for template preparation can be any type of patient specimen, including blood, body fluids, whole tissue, scrapings, washes, etc. Each specimen type can bring its own problems regarding template preparation. Components in blood specimens, such as hemoglobin and lactoferrin, as well as the preservatives or anticoagulatives in specimen collection such as EDTA and heparin, can inhibit downstream steps such as PCR, if they are not removed during template preparation (10). Tissue specimens that are only lightly colonized with an infecting fungus can swamp template preparations with host nucleic acids, proteins, etc such that fungal material cannot be purified or cannot be detected against the excess background of host material. Furthermore, tissue specimens too often can be limited in amount, preventing multiple preparations. Alternatively, depending on the source, specimens from non-sterile sites such as trauma, sputum, or bronchial-lavage can be contaminated with other fungi that are not invasive, but nonetheless, could show up in assays that are pan-fungal in nature (11, 12). Finally, there is the problem of formalin-fixed paraffin-embedded (FFPE) tissue specimens, which confer difficult challenges in spite of the wide availability of commercial kits that claim to be effective for processing these types of specimens. These specimens often make molecular detection difficult because nucleic acids can be cross-linked or fragmented

during the fixation, leading to non amplification by PCR (13). They also can be externally contaminated (14), in addition to the ever-present problem of efficiently breaking the fungal cell walls once they have been suitably deparaffinized. Unfortunately, fixed specimens are among the most valuable fungal diagnostic specimens so there is substantial opportunity for template preparation improvement.

As trivial as template preparation seems, this step may be the most important area of molecular diagnosis to develop and standardize. Unfortunately, because of the diverse nature of fungal cell walls, with yeasts generally being easier to lyse than moulds, it has been difficult to devise a "one size fits all" method. Until this challenge is overcome, new technologies will not be able to assume most of the diagnostic burden in a clinical laboratory.

4. DIAGNOSTIC STRATEGIES

Application of molecular biology to fungal diagnosis has drawn from many areas, which has resulted in the successful use and continued development of numerous molecular platforms for fungal identification. The stringent conditions that clinical microbiology laboratories operate under combined with the multiple isolate sources (pure culture, human specimens, fixed tissue, etc) pose difficult challenges to the widespread use of molecular biology in clinical mycology. However, the numerous working assays (Table 1) and the continual development of new technologies offer great promise for one or more platforms to become standard equipment in a clinical laboratory.

4.1 PCR

Among the most basic of all molecular assays, and the most common molecular technique used by mycologists, is PCR. This technique is roughly 30 years old and is the workhorse of molecular biology,

with almost limitless uses (15). There are countless diagnostic uses, and importantly, an extensive array of variations of the basic technique that have specific applications. The most basic PCR strategy is conventional PCR, which consists of two primers that anneal to complimentary target sequences. *Taq* polymerase, in addition to the appropriate buffer mix and template DNA, is used with a thermocycler to yield a product from a suitable starting template. This product can be the endpoint of the assay when visualized on an ethidium bromide gel, or it can be digested to yield specific restriction patterns that can be informative. Typically, conventional PCR assays have as an endpoint the presence or absence of an amplification product and almost always need to utilize specific primers that typically amplify a single or very narrow range of fungi. Size markers are used to confirm that the product is from the correct target since the amplicon size is usually known based on primer position. While degeneracy or conserved target sequences can extend the species target range, this extension can defeat the purpose if the goal is species identification. In some cases the goal can be detecting the presence of a specific gene or allele, which could carry pathogenic or drug susceptibility consequences when it is present. Continued research has resulted in many variations that have made the technique more powerful, more sensitive, cheaper, and more creative in its applications.

The most common platform of non-conventional PCR for fungal detection is quantitative real time PCR (qPCR), which offers a number of advantages over the older gel-based conventional PCR approach. The major advantage of qPCR is that it can be used to quantitate fungal colony forming units, or the equivalent, and it is generally faster since the technique incorporates a target-specific, fluorescently labeled probe, or a general non-specific dye that preferentially binds double stranded DNA. The small amplicon size in qPCR, sometimes less than 100 bp, greatly decreases cycling time as amplicons can be 1/10 the size of conventional PCR amplicons. Labeled probes confer the extreme specificity that qPCR is known for, although they add substantially to reaction costs compared to a non

specific DNA binding dye such as SYBR Green. However, suppliers of qPCR probes often allow web-based, specific assay design using algorithms that precisely match the two PCR primers and probe in a single reaction, which can be purchased as an individual assay, often as a ready-to-go PCR kit complete with enzyme and other reaction components. The programs work on a user-supplied target sequence that is simply pasted into the website to yield each assay with individual components tailored to the most efficient amplification of the target. These programs can be proprietary, available on the internet, or part of desktop molecular biology programs.

Reactions are performed in a dedicated thermocycling instrument that can detect multiple dyes that fluoresce at different wavelengths, which can allow multiplexing in a single tube. There are various classes of fluorescent dye technologies for target detection, depending on the need. Although an FDA-approved fungal diagnostic assay using qPCR technology has not reached clinical laboratories, qPCR is used extensively for the detection and quantitation of other microbes, consequently, it is only a matter of time before a specific fungal assay is routinely used in the clinical laboratory. However, it has been applied to virtually all of the major and more common human fungal pathogens and is frequently a part of most research laboratories.

There are a number of conventional PCR variations that have displayed clear value in the area of diagnostics. Some of them are moving away from the thermocycler platform and into isothermal amplification, which has the major advantage of greatly reducing instrumentation costs because reactions are run at a single temperature that can be reached with a heating block. Nucleic Acid Sequence Based Amplification (NASBA), is a constant temperature (41°C) transcription-based amplification method that uses RNA as a template and a combination of RNase H and T7 RNA polymerase as enzymes. There are numerous advantages of this technique as it amplifies RNA templates, which can be present in great abundance for some targets (i.e., rRNA). Because RNA is the

template, exogenous amplification of contaminating DNA is eliminated. The constant temperature and single tube reaction also reduce contamination possibilities. A commercial version of this technology (AccuProbe, Hologic Gen-Probe Inc., San Diego, CA) has been available for a number of years and can be used to detect *Histoplasma capsulatum*, *Coccidioides immitis*, and *Blastomyces dematitidis* (16-18).

A second type of isothermal PCR is called Loop Mediated Isothermal Amplification (LAMP) (19). The key component of this reaction is a DNA polymerase derived from the bacterium, *Bacillus stearothermophylus*. Reactions can be completed within an hour and are performed isothermally at a temperature range of 60-65°C. In addition to the isothermal temperature and relatively fast turnaround time, LAMP is resistant to exogenous contaminants due, in part, to the requirement for four primers. Therefore, template nucleic acids do not need to be highly purified, allowing cruder, "dirty" preparations to be used, however, the large number of primers can add complexity to primer design compared to traditional PCR reactions that utilize two primers (20). There are multiple product detection methods, which vary from ethidium bromide stained gels, to turbidity, as well as fluorescence. LAMP has been successfully used to detect a number of fungi including *Fusarium* spp.*, Cryptococcus spp.,* and *Aspergillus* spp. (21-24).

Rolling Circle Amplification (RCA) is a third type of isothermal amplification technology that can result in the massive amplification of target sequences (~10^9 fold) (25). This technology utilizes bacteriophage Phi29 DNA polymerase to amplify a DNA template. RCA is highly sensitive and specific, and can be used to discriminate single nucleotide polymorphisms. The technology utilizes a circular template to generate linear amplification products, which can be detected fluorescently using a variety of different probe chemistries. This sensitivity is particularly important for fungal genera that can be difficult to distinguish at the species level, even with molecular methods, due to highly

conserved target sites (26). The method is generally refractory to inhibitory compounds present in a sample that would normally inhibit other types of PCR reactions. RCA can also be easily multiplexed in contrast to conventional PCR so that multiple targets can be detected simultaneously. RCA has been used to detect a variety of fungi including *Penicillium marneffei*, *Fonsecaea* spp., and *Exophiala* spp. (27-29).

In addition to these technologies, there are various other PCR-based methods that have various applications in diagnostic mycology. For example, PCR can be used for some aspects of epidemiology. For fungi, this application can be extremely important because in many cases, little is known about the infecting fungus. A major PCR-based application in fungal epidemiology is Random Amplification of Polymorphic DNA (RAPD). RAPD is a fingerprinting method that utilizes single, short oligonucleotides (~10 bp) in a PCR reaction typically at low annealing temperatures. PCR products can be produced when two oligonucleotides anneal close enough together on complementing DNA strands to produce a PCR product. Running the PCR reaction on an agarose gel followed by staining reveals an isolate-specific pattern that can be used to distinguish two isolates from one another, depending on the oligonucleotide. The discriminating power of the assay relies on multiple products being produced in a single reaction. Genetically unrelated strains are predicted to have different RAPD patterns depending on how fast their genomes evolve, geographic isolation from each other, and the frequency of the primer site in the genome. The greatest advantage of RAPD as an epidemiological tool is that nothing needs to be known about the genome sequence of the organism. The primer choice can be made empirically by testing single primers on one isolate and selecting the primer that yields the most bands for further testing on unrelated strains. For some fungi, such as *Cryptococcus neoformans*, this method has been studied extensively and has been developed into a strain typing tool based on PCR pattern (30). The method can be highly susceptible to variation, even within laboratories, and is therefore not

generally used as an identification tool. However, it has tremendous utility as a rapid way to investigate outbreaks to determine if infections are from the same or unrelated strains.

Repetitive sequence PCR (rep-PCR) is both an epidemiologic and diagnostic tool that can, in some cases, identify as well as discriminate individual fungal strains (31). The method is PCR-based and relies on the high frequency of repetitive elements found in some microbial genomes, including bacteria and fungi. Using a large collection of stock primers in a semi automated platform, the method can be used to fingerprint fungi as well as identify some species of fungi by searching the generated fingerprint pattern against a reference library (32, 33). The method is well advanced and has already been commercialized (Diversilab, bioMérieux, Inc., Durham, NC).

4.2 Sequencing

While live culture is generally considered the gold standard of fungal diagnosis, arguably, the "gold ring" of molecular diagnostics should be a system that can identify any fungus, independent of any accompanying information (patient history, symptoms, imaging, morphology, biochemistry, or serology results, etc), using a single assay. While there are a number of methodologies that may be able to fulfill this possibility, the technology that is farthest along is sequence-based identification. Key factors that have enabled sequence-based identification to be as powerful as it is are the easy access to public databases, such as GenBank, and the search algorithms such as the BLAST programs (34) that enable users to query the database with an unknown sequence. Inexpensive sequencing, through advances in instrument technology and chemistry, has enabled the generation and deposit of sequences from countries all over the world leading to both a deep and diverse database of easily searchable fungal sequences. The field of bioinformatics has made it possible to determine which sequences are the most informative and discriminatory with regard to species identification. Bioinformatics has

advanced hand in hand with the completion of genome sequences, of which hundreds of fungal genomes are now complete. Next generation sequencing will greatly accelerate genome sequencing and may someday be as fast and inexpensive as obtaining a single gene sequence is today.

Sequence-based identification of fungi evolved into a powerful diagnostic tool with the deposit of increasing numbers of ribosomally derived sequences (18s subunit, internal transcribed spacer regions 1 &2, 5.8s subunit, 28s subunit, and the intergenic region). These sequences were important for fungal phylogeny and drove diagnostic mycology into the molecular era, however, as the deposits accumulated, both conserved and variable regions were identified. The existence of these regions allowed the design of primers to conserved regions, which enabled PCR amplification and sequencing of an unknown fungus through universal primers that could anneal to the conserved sites, and amplify the unknown intervening regions. The resultant amplicons contain the conserved priming sites in addition to the informative intervening regions, which enabled identification, often to the species level. Two of the most common pairs of primers are the ITS1 and ITS4, and the NL1 and NL4 primers (Fig. 2) (35, 36). There are many other primers that are suitable for amplifying informative regions, however, these primers were used to deposit many of the fungal sequences into GenBank that are useful for diagnostic purposes. These two pairs amplify informative regions containing the internal transcribed spacer regions (ITS1 and ITS2) as well as the D1/D2 region of the 28s or large ribosomal subunit. Both regions are informative when searching GenBank, although the region amplified by the ITS1 and ITS4 primers is generally more variable than the region amplified by the NL1 and NL4 primers. Furthermore, the ITS region contains two individually informative regions, the ITS1 and ITS2 regions, which are separated by the 5.8s subunit. A third potential amplicon, in addition to the ITS and D1/D2 regions is the intergenic sequence, sometimes referred to as the IGS region (Fig. 2). This region separates the 28s and 5s subunits and is extremely variable. However, it is generally too long for

universal primers to be used in a PCR reaction, although it can be used to distinguish specific species, but has not been applied to general fungal molecular identification.

In addition to the conserved and near universal PCR priming sites, a second important factor for targeting the rDNA for molecular identification is copy number. Eukaryotic ribosomal subunits are typically present in multiple copies that can exceed single copy targets by 10-100x. This increased copy number confers greater sensitivity for PCR reactions, which can be extremely important when template is not prepared from pure culture, but instead, from tissue specimens, body fluids, or other samples that may have a low or limited amount of organisms. In addition to some of the multiple mitochondrial targets, the multicopy nature of these targets is indispensable for limited amounts of cellular material, as is often the case when patient specimens must be tested directly.

When ribosomal targets are not specific enough and cannot distinguish isolates at the species level, additional sequencing targets are commonly used and typically are organism-dependent. These targets are usually single copy genes that are generally highly conserved, but not conserved enough to allow universal primers. In fact, degenerate primers may sometimes need to be used in order to amplify all desired species in a genus. Among the most common single copy genes and the fungi they are used for are β-tubulin for the discrimination of *Aspergillus* spp., *Phaeoacremonium spp., Penicillium* spp., elongation factor 1 alpha for discrimination of *Fusarium* spp., cytochrome oxidase subunit 1 for discrimination of *Penicillium* spp., calmodulin for discrimination of *Pseudallescheria* spp., *Sporothrix*, and *Scedosporium* spp., glyceraldehyde-3-phosphate dehydrogenase for discrimination of *Cochliobolus* spp*., Curvularia* spp., and *Bipolaris* spp. and actin for discrimination of Rhizopus spp., *Stachybotrys* spp., and *Trichophyton* spp. (37-47). In many cases multiple genes may need to be used to discriminate different species depending on the genus. Presently there are no specific criteria that are used to determine what target should be used to discriminate different species. Decisions are typically

made through individual laboratory experience and publications describing phylogenetic studies that have included multiple species and multiple gene sequences. Finally, one of the great challenges to sequence-based diagnostics has been how to best standardize the methods used to arrive at an identification, and fit these guidelines within the normal function of a clinical laboratory. Presently, the most useful guidelines are provided by the Clinical Laboratory Standards Institute, which describe in detail sequence-based identification of fungi and the targets that can be used (48). In spite of the multitude of targets and the potential to recover an informative sequence from any fungus, the most important caveat to sequence-based identification is that the value of the sequence data is completely dependent on the database it is searched against. The public databases, which are extraordinarily deep, are the best sources for rare fungi since data are collected from throughout the world for a broad spectrum of fungi, often with extensive redundancy. However, because anyone can deposit data, the error rate of data in the public databases is so high that databases such as GenBank are unlikely to meet the requirements of being a sole source for molecular identification under certified conditions. Instead, for the highest quality data, biocurated databases are needed that are closed to unauthorized deposits and contain only reference sequences derived from fungi with confirmed identity, such as culture collection isolates. There are a number of these databases with some, such as the Microseq Identification System (Applied Biosystems, Foster City, CA), even being commercialized. However, while the data are highly dependable, the number of unique species is going to be limited to fungi that are most commonly encountered in patients.

4.3 Arrays

In spite of the power of PCR-based technologies, even with multiplexing, it is difficult to incorporate a large number of potential identities into a single reaction. To increase the potential number of species

that can be identified in a given assay, various array-based technologies have been developed that can expand the number of species that can be identified in a single assay by one to two orders of magnitude.

Arrays can be formatted in a number of different platforms and generally function by anchoring a set of known targets, in the form of a nucleic acid sequence, to some type of supportive matrix such as a bead, glass chip, or membrane. The unknown specimen is then hybridized to the array and a read out signal is generated, which can be decoded and matched to the target identity. The simplest arrays consist of spotting targets onto membranes using the Southern blotting platform developed years ago (49). Numerous arrays of this type have been used to identify fungal species (50, 51).

Miniaturization of the arrays into a microarray format by high-density spotting enables a massive amount of spots, numbering in the thousands or tens of thousands, to be interrogated by the sample. However, the drawback is that these assays can be insensitive without an amplification step and can be slower than PCR due to the kinetics of DNA:DNA hybridization, which can take many hours. Decoding of the reaction—identifying the spot that the unknown hybridized to can require specific instrumentation and advanced software. Furthermore, each spot carries a species-specific reagent that in turn carries a cost to be incorporated into the array. For *C. albicans*, which is frequently recovered from clinical specimens, the assay is cost-effective. However, incorporating hundreds of other spots corresponding to fungi that are known to be pathogenic, but are so rare that they may never be encountered in a clinical laboratory, adds to cost but with questionable return.

Although the chips can be prepared robotically with extreme precision, the cost per assay can be quite expensive compared to alternatives. Scaling this process down using nanotechnology to create Lab-On-A-Chip assays that employ microfluidics is a newer approach to array technology that offers great promise due to the smaller reaction volumes (microliters) and user-friendly format more

conducive to a clinical laboratory. Additionally, engineering cartridges containing the targets and marrying these cartridges to an instrument can greatly simplify and standardize the hybridization reactions. Large-scale application to any unknown fungal isolate has been slow to develop using arrays, however, focused use at the genome level has achieved some success. For example, Aittakorpi et al., have used microarrays to identify *Candida* spp. in cases of fungemia using the Prove-It, Sepsis microarray, which was derived from a microarray-based approach for identifying bacterial sepsis (52, 53).

Finally, bead-based arrays that combine the power of multiplexing and the simpler platform of liquid suspension have been successful in identifying a number of fungal species. An 11-plex bead array was used by Buelow et al., to identify common respiratory-associated fungi using the Luminex platform (54). Babady et al., used the same platform to design a Luminex-based assay to identify 23 fungi commonly found to cause invasive infections in the immunocompromised while Balada-Llasat et al., used the Luminex platform to identify yeasts from blood cultures (55, 56). These assays are targeted approaches to identify anticipated groups of fungi. The Luminex platform can multiplex almost 100 fungal analyte targets, which potentially could detect most of the commonly encountered fungi.

4.4 PNA FISH

Peptide Nucleic Acid Fluorescence in Situ Hybridization (PNA FISH) (AdvanDX, Woburn, MA) was adopted into some clinical microbiology laboratories to discriminate *C. albicans* from non-*C. albicans* directly from positive blood cultures (57, 58). The assay employs PNA probes to target specific rRNA sequences in a highly sensitive and specific FISH assay that allows the technologist to view whole cells of the target pathogen (59). The rRNA targeting brings enhanced sensitivity due to the abundance

of the target. Because the probe is sequence-based, it can be highly discriminatory due to differences in target sequence, yet downstream manipulation of data is not required since the assay is read visually by detection of fluorescent cells. In fact, the probe is able to discriminate *C. albicans* from *C. dubliniensis*, which is nearly impossible to do biochemically (60). This assay requires limited sample preparation since cells do not need a prior extraction step, and quick visual results within 90 minutes are usually obtainable. The main drawback of the assay is based on the need for cells to be permeable to the probe since it needs to enter the cell. For *Candida* spp. permeability is not a major problem, however, filamentous fungi are much less amenable to this assay as a diagnostic tool since fungal hyphae are generally not as permeable as yeast cells.

4.5 MALDI-TOF

Culture is the "gold standard" of diagnostic mycology, however, waiting for cultures to grow and differentiate can take days, or even weeks for some fungi such as *Histoplasma* sp.. As a result, physicians may treat patients for days without knowing whether their patients are actually infected or the identity of the organism causing their patients' illness. Matrix Associated Laser Desorption Ionization-Time of Flight (MALDI-TOF) is a recent development in the clinical microbiology field that is rapidly changing the routine diagnostics field because of the speed with which samples can be run through the instrument (61).

MALDI-TOF uses the unique protein fingerprint of each species to give identification in a few minutes if the unknown sample has a matching reference spectra in the library that comes linked to the instrument. After a quick sample preparation, fungal cells are mixed with a UV absorbing matrix and dried on the steel target plate. The dried preparations are loaded onto the MALDI-TOF instrument and are subsequently exposed to laser pulses, resulting in energy transfer from the matrix to the analyte

creating a “charged vapor” of the biomolecules of interest (62). The particles in this vapor are accelerated and separated under the influence of an electric field, which requires different times to reach the detector and is referred to as the time of flight (63). The proteins are then separated based on their mass/charge ratio, which creates a unique pattern of peaks or fingerprints at the detector, also known as the mass spectrum. The resulting spectra of biomolecules are unique and species-specific. The spectrum of each organism is compared to a library of reference spectra within the instrument, leading to an identification within minutes as opposed to days or weeks for some fungi (61).

The rapid turnaround time and minimal cost for consumables per specimen compared with conventional identification methods has resulted in MALDI-TOF being increasingly used in clinical laboratories worldwide. Adoption of this technology in clinical microbiology laboratories across the US will decrease the time-to-identification of fungal pathogens by an average of 1-2 days and decrease laboratory costs. The MALDI-TOF instrument has an upfront cost of $213k (including sales tax and first year service contract) and ~$35k to operate annually, depending on instrument manufacturer. This initial capitol outlay is substantial, however, the cost per sample can be less than 5 cents, which is a significant reduction compared to the cost to run PCR, biochemical methods, or PNA-FISH.

Several platforms are available; a system from Bruker Daltonics (Billerica, MA), which includes a mass spectrometer along with the “Biotyper” software and database. Another system uses a Shimadzu Axima Assurance mass spectrometer (Columbia, MD) Launchpad software and the AnagnosTec GmbH (SARAMIS) database. This system was recently acquired by bioMérieux, and is being redeveloped and called “VITEK MS.” These two systems exclusively use cell lysis on the target plate (without off-plate extraction).

The great advantage of MALDI-TOF is that it addresses a number of short-comings with other molecular methods. Template extraction, while not yet completely universal, is still easy and relatively

labor-free compared to other methods. However, once this step is done, the sample is ready for analysis as there are no further manipulations needed. There is no downstream analysis after the sample has been run as the instrument will call the identification based on generated spectra, which is compared to the reference library of spectra. Additionally, the instrument can analyze other microbes with little variation in sample preparation, which opens the door for standardization. Drawbacks include large capitol start up costs, and annual expenditures for service. The instrument is only as good as the reference library, and although users can add to the library, it would take years to make a spectral library as deep as the sequence databases. Furthermore, libraries are proprietary as there is no public database of spectra. Another drawback is the need to culture the organism prior to performing the test, so arguably, the analysis turnaround time benefit is mitigated by culture outgrowth delay. Validation studies have shown that some organisms require repeat analyses, and additional processing. The acceptable score cutoffs also vary between organisms (61). Some closely related fungal organisms cannot be differentiated, and sporulation or different morphologic growth phases may present a challenges for identification since each morphology may require a different set of proteins resulting in variable spectra. Finally, these systems are not yet approved for use in the US by the Food and Drug Administration (FDA). The current lack of FDA approval of any MALDI-TOF MS system for organism identification limits widespread use in the United States.

In spite of these areas in need of improvement, MALDI-TOF is an exciting new technology with many promising advantages. It is automated, doesn't require specific expertise in mass spectrometry, has a rapid turnaround time, and high throughput capability. It is associated with a low exposure risk due to sample inactivation and is cost effective with high inter-laboratory reproducibility. The broad applicability covering all types of bacteria, mycobacteria and fungi makes the instrument more palatable to microbiology laboratory budgets.

5. *SUMMARY*

Diagnostic mycology has a great need for molecular approaches to fungal identification. While there is no doubt that classical mycology diagnostic methods will not become obsolete anytime soon, the field needs to recognize that medical mycologists with diagnostic skills are not being trained in great numbers. Consequently, clinical microbiology laboratories are at great risk for not having the expertise to handle fungal identification. While not a substitute for classically trained mycologists, adding a molecular component to diagnostic mycology can greatly ease the loss of these skills and offer some opportunities for synergism. The increasing degree of immunosuppression, aggressive nature of modern medical treatment, and an aging population are just a few of the risk factors that contribute to infections with a broadening spectrum of fungi never seen before in the clinical laboratory. Frequent case reports of new fungal pathogens combined with regular discoveries of new species of fungi that are also capable of infecting humans clearly demonstrates that the field of clinical mycology needs to expand, not contract, with regard to the skills that can be applied to fungal identification. While there is no clear path for training a new generation of medical mycologists without the support of these programs at academic institutions, new technology, instrumentation, and strategies can offset pending losses of experienced mycologists. Therefore, from a strategic perspective, these advances should be integrated into the clinical laboratory whenever possible to enhance the possibility that one or more may emerge as a viable alternative or addition to classical identification methods.

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FIGURE LEGENDS

Fig. 1. The relationship between yield, number of steps, and type of fungal template preparation method. The greatest yield comes from laborious methods that require numerous steps, including organic extractions. In contrast, the easiest methods yield the least DNA, which is typically lower in quality and more likely to be contaminated with cellular material.

Fig. 2. Organization and primer location of the fungal ribosomal genes. The ITS region, generally the most informative, can be amplified by the ITS1 and ITS4 primers, and is located between the end of the 18s ribosomal sub unit and the 28s ribosomal subunit. The D1/D2 region if found within the 28s ribosomal subunit towards the 5' end and can be amplified with the NL1 and NL4 primers. The IGS region is also quite variable and is typically but not always found between the 28s and 5s subunit. However, the size of this region is generally too large to amplify in a PCR reaction. Not drawn to scale. Open arrowheads indicate primer sites.

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Table 1. Comparison of different diagnostic platforms.

Method	Capitol Cost	Technical Expertise	Assay Cost	Turnaround Time	Downstream Analysis	Labor	Sensitivity	Specificity
PCR	Low	Lowest	Low	Low	Low	Low	High	High
qPCR	Medium	Low	Low	Low	Low	Low	Highest	Highest
NASBA	Lowest	Lowest	Low	Low	Low	Low	High	High
LAMP	Lowest	Lowest	Low	Low	Low	Low	High	High
RCA	Lowest	Lowest	Low	Low	Low	Low	Highest	Highest
REP-PCR	Low	Low	Low	Medium	Medium	Low	High	Medium
RAPD	Low	Lowest	Low	Medium	Low	Medium	Lowest	Lowest
Sequencing	Highest	Highest	Low	Medium	Medium	Medium	High	High
Array	High	Low	High	Medium	Low	Low	High	High
PNA-FISH	Medium	Low	Low	Low	Lowest	Lowest	High	Highest
MALDI-TOF	Highest	High	Lowest	Lowest	Lowest	Lowest	Medium	High

PCR; Conventional Polymerase Chain Reaction, qPCR; quantitative real time PCR, NASBA; Nucleic Acid Sequence Based Amplification, LAMP; Loop Mediated Isothermal Amplification, RCA; Rolling Circle Amplification, REP-PCR; Repetitive sequence PCR, RAPD; Random Amplification of Polymorphic DNA, PNA-FISH; *Peptide Nucleic Acid Fluorescence in Situ Hybridization, MALDI-TOF;* Matrix Associated Laser Desorption Ionization-Time of Flight. Capitol costs are generally defined as high (~$100k or more) to low (<$1000). Technical expertise is defined as high (specialized training or background) to low (basic laboratory skills with no special training). Assay cost is defined as high (>$50) to low (<$5). Turnaround time is defined as high (>5 hrs) to low (<3 hrs). Downstream analysis is defined as high (extensive data manipulation or transformation) to low (immediate read out). Labor is defined as high (>3 hrs preparation) to low (<1 hr preparation). Sensitivity is defined as high (detection of <100 CFU) to low (>10^4). Specificity is defined as high (species level discrimination) to low (genus or lower order).

Figure 1

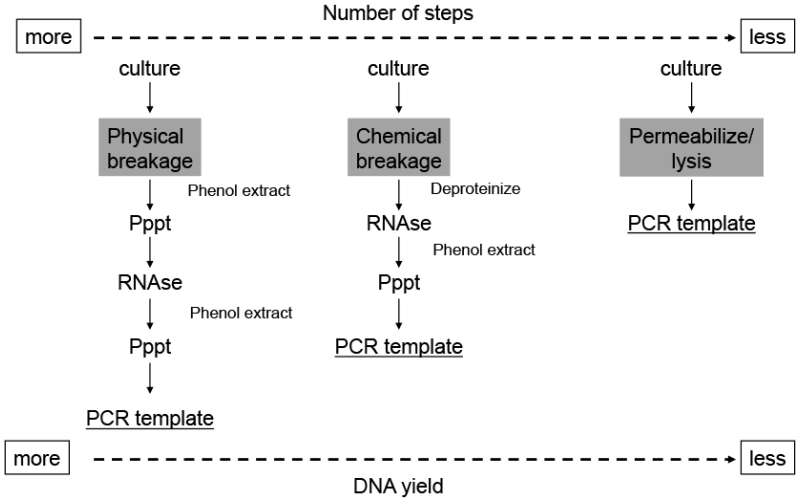
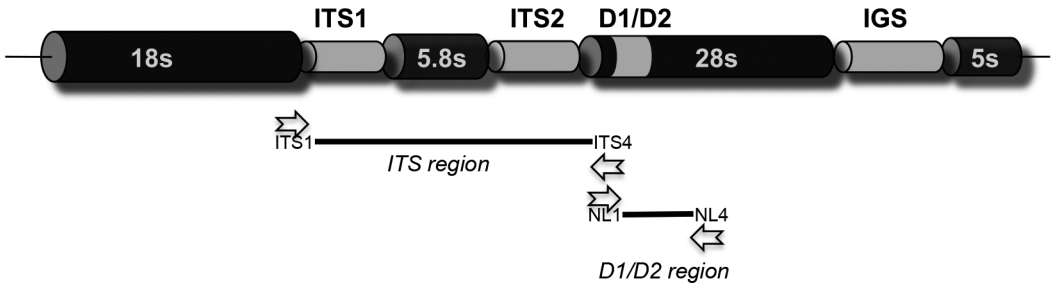

Number of steps
more
less
culture
culture
culture
Physical breakage
Chemical breakage
Permeabilize/ lysis
Phenol extract
Deproteinize
Pppt
RNAse
PCR template
Phenol extract
RNAse
Pppt
Phenol extract
Pppt
PCR template
PCR template
more
less
DNA yield

Figure 2.


ITS1
ITS2
D1/D2
IGS
18s
5.8s
28s
5s
ITS1
ITS4
ITS region
NL1
NL4
D1/D2 region

Original article

A universal DNA extraction and PCR amplification method for fungal rDNA sequence-based identification

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Summary

Accurate identification of fungal pathogens using a sequence-based approach requires an extraction method that yields template DNA pure enough for polymerase chain reaction (PCR) or other types of amplification. Therefore, the objective of this study was to develop and standardise a rapid, inexpensive DNA extraction protocol applicable to the major fungal phyla, which would yield sufficient template DNA pure enough for PCR and sequencing. A total of 519 clinical and culture collection strains, comprised of both yeast and filamentous fungi, were prepared using our extraction method to determine its applicability for PCR, which targeted the ITS and D1/D2 regions in a single PCR amplicon. All templates were successfully amplified and found to yield the correct strain identification when sequenced. This protocol could be completed in approximately 30 min and utilised a combination of physical and chemical extraction methods but did not require organic solvents nor ethanol precipitation. The method reduces the number of tube manipulations and yielded suitable template DNA for PCR amplification from all phyla that were tested.

Key words: 18S RNA, 28S ribosomal DNA, diagnostic, ITS, D1/D2.

Introduction

The frequency of fatal mycoses has escalated over the last two decades due to the increasing number of populations at risk for infection.[1,2] Those at increased risk are patients with compromised immune systems such as those with leukaemia, AIDS, stem cell and organ transplants, cancer and other patients subjected to treatment or disease that suppresses the immune system.[2] Systemic mycoses have been known to decrease the chance of recovery for these patients when they are not diagnosed and treated in a timely manner.[3–5] Because delayed diagnosis of a fungal infection can lead to severe and often life-threatening consequences[6,7] early and accurate diagnosis of fungal infections will help facilitate prompt and appropriate treatment leading to better patient outcomes.[8,9]

Culture-based methods used to identify fungi have often relied on laboratory identification by micro- and macroscopic morphology as well as biochemical tests. In most cases, traditional phenotype-based methods can be successfully used to identify common fungi; however, these methods have been shown to provide poor diagnostic sensitivity, have long turnaround times and can lack specificity for a species-level identification.[10] Delayed diagnosis and treatment can be minimised with the development of more rapid diagnostic techniques for identification of fungal infections.[11] Although conventional methods are still in practice, there has been an increase in the use of molecular approaches for fungal identification.[5] Polymerase chain reaction (PCR) and sequence-based assays offer overwhelming advantages over conventional culture techniques for fungal identification,

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including the detection of unculturable fungi, rapid analysis, reproducible results and the ability to identify non-differentiated or sterile fungi.[12] Importantly, rarely encountered fungi can often be identified using DNA sequencing due to the massive amount of information in public databases.

The gene targets most commonly used for sequence-based fungal identification are the internal transcribed spacer regions ITS1 and ITS2, which are variable regions (the ITS region) located between conserved genes encoding the 18S, 5.8S and 28S ribosomal subunits.[13] The ITS region can be amplified from a wide range of fungi using the ITS-1 and ITS-4 primers.[14] A second variable region within the rDNA cluster, called the D1/D2 region, is located towards the 5′end of the large nuclear ribosomal subunit (28S rDNA).[8,15] The D1/D2 region can also be amplified from a diverse array of fungi using primers NL-1 and NL-4.[15] While this region is also informative, it tends to be less variable than the ITS region, although there are exceptions.[16] The conserved nature of the flanking ends of the D1/D2 and ITS regions serve as the priming sites for amplification, while the variable nature of the internal regions can be species specific.[8,15] However, one of the greatest advantages of using the ribosomal locus as a target for PCR amplification is the increased copy number of these genes in most fungi, as much as 10–100 times that of single copy genes, which translates into greater sensitivity.[17–19]

In instances, where ribosomal targets are not sufficiently sensitive for the discrimination of closely related species, additional loci can be sequenced. Examples of second loci include genes such as β-tubulin for identification of individual species within the various *Aspergillus* sections,[20,21] or the orotidine monophosphate pyrophosphorylase (*URA5)* gene for the identification of *Cryptococcus neoformans* to the variety level.[21] Other genes, such as translation elongation factor 1 alpha (*EF1*α), are routinely used in our laboratory depending on the isolate. While these genes tend to be conserved structural genes, they are not conserved enough at the DNA level to offer universal priming sites, even with degenerate primers, consequently primer choice may require some preliminary information about the possible genus of the isolate. None the less, utilisation of the ITS, D1/D2, or other loci as PCR and sequencing targets can provide rapid, accurate identifications within 8 h, which is significantly shorter than live culture procedures.[22]

The first step in virtually all molecular-based diagnostic assays requires the isolation of template nucleic acid free of possible contaminants that can inhibit downstream steps in amounts sufficient enough for downstream assays. Fungal DNA extraction protocols can often be time consuming because of the need to lyse the cell wall and remove contaminating proteins and carbohydrates. These procedures typically require laborious protocols and/or hazardous chemicals[23–26] and can be among the most time-consuming steps of sequence-based identification. Spin column or other DNA binding matrices can replace organic extractions in some cases; however, the DNA binding material can sometimes be contaminated with fungal DNA.[10] The shortcomings of these protocols have resulted in a lack of a simple, inexpensive, universal procedure suitable for the efficient isolation of DNA from phylogentically diverse fungi. Therefore, the objective of this study was to develop a simple protocol for DNA extraction that would yield template DNA suitable for PCR amplification from all fungi.

Materials and methods

Strains and media

The strains used in this study are listed in Table 1. These isolates (168 in total) were culture collection isolates with established identities and were used to confirm the robustness and accuracy of the assay. Additional strains (351 in total) were obtained from the UTHSCSA Fungus Testing Laboratory. Potato Dextrose Agar (Difco, Inc., Detroit, MI, USA) plates were used to culture moulds. Yeast isolates were grown on Yeast Peptone Dextrose agar (2% dextrose, 2% peptone, 1% yeast extract and 2% agar). Growth conditions, such as incubation temperature or media composition (such as adding corn oil to the medium for some *Malassezia* species), were varied as needed. However, most isolates were grown at 30 °C.

Preparation of template DNA

Template preparation worked best when DNA was prepared from rapidly growing cells, which was typically an 18- to 24-h culture. A small amount of hyphae or yeast cells were collected from culture plates using a disposable 1 μl/10 μl inoculating loop (Fisher Scientific, Inc., Pittsburgh, PA, USA), to recover a loopful of colony material. Due to the nature of the growth of filamentous fungi, hyphal material from some mould specimens could not be scraped off the agar to prepare sufficient template DNA due to growth below the agar surface. In these cases, the loop was used to remove small pieces of agar material with hyphal growth, which were then broken apart by rubbing and rolling

Table 1 Strains used in this study.

Fungal isolates	Culture collection ID
Absidia corymbifera (Lichtheimia corymbifera)	NRRL 6252[1]
Absidia glauca	CBS 101.08[2]
Acremonium kiliense (Sarocladium kiliense)	ATCC 34716[3]
Acremonium strictum (Sarocladium strictum)	CBS 646.75
Actinomucor elegans	ATCC 22814
Actinomucor elegans var. kuwaitiensis	R-831[4]
Arthroderma fulvum	ATCC 16445
Arthroderma vanbreuseghemii (Trichophyton interdigitale)	ATCC 24960
Arthrographis kalrae	ATCC 18434
Aspergillus candidus	CBS 102.12
Aspergillus flavus	CBS 569.65
Aspergillus fumigatus	CBS 113.26
Aspergillus glaucus (Eurotium herbariorum)	CBS 516.65
Aspergillus granulosus	CBS 119.58
Aspergillus lentulus	NRRL 35551
Aspergillus niveus	CBS 444.75
Aspergillus ochraceopetaliformis	R-4478
Aspergillus oryzae	CBS 108.24
Aspergillus restrictus	CBS 118.33
Aspergillus sclerotiorum	CBS 632.78
Aspergillus sydowii	CBS 593.65
Aspergillus thermomutatus	CBS 208.92
Aureobasidium pullulans	CBS 100524
Blakeslea trispora	ATCC 11517
Bjerkandera adusta	ATCC 16996
Byssochlamys nivea	CBS 100.11
Candida albicans	CBS 562
Candida blankii	CBS 1898
Candida boidinii	CBS 2428
Candida caseinolytica	CBS 342.67
Candida catenulata	CBS 565
Candida chilensis	CBS 5719
Candida cylindracea	CBS 6330
Candida diversa	CBS 4074
Candida dubliniensis	CBS 7987
Candida krusei (Pichia kudriavzevii)	CBS 2050
Candida membranifaciens	CBS 1952
Candida metapsilosis	ATCC 96144
Candida orthopsilosis	ATCC 96139
Candida parapsilosis	CBS 604
Candida stellata	CBS 157
Candida tropicalis	ATCC 4563
Capronia coronata	CBS 1232
Cephalotheca foveolata	ATCC 1953
Chrysosporium vallenarense	ATCC 64421
Cintractia sorghi-vulgaris	CBS 2356
Cladosporium cladosporioides	ATCC 18831
Cladophialophora minourae	R-4222
Clavispora lusitaniae	ATCC 200953
Climacodon septentrionalis	CBS 1209
Clydaea vesicula	JEL 369[5]
Cokeromyces recurvatus	ATCC 2564
Conidiobolus lamprauges	ATCC 12585
Coprinellus domesticus	ATCC 26829
Coprinus domesticus	ATCC 26829

Table 1 (Continued)

Fungal isolates	Culture collection ID
Cryptococcus neoformans var. neoformans (serotype A)	CBS 882
Cryptococcus neoformans var. gattii (serotype B)	ATCC 64062
Cryptococcus neoformans var. gattii (serotype C)	ATCC 32269
Cryptococcus neoformans var. neoformans (serotype D)	ATCC 90126
Debaromyces hansenii var. hansenii (Debaryomyces hansenii)	CBS 767
Dipodascus capitatus	CBS 197.35
Emericella rugulosa	CBS 133.60
Emmonsia parva	ATCC 10784
Eurotium repens (Aspergillus pseudoglaucus)	CBS 529.65
Eurotium rubrum (Aspergillus rubber)	CBS 530.65
Exophiala alcalophila	ATCC 48519
Exophiala aquamarina	R-1181
Exophiala dermatitidis	ATCC 201305
Exophiala dopicola	CBS 537.94
Exophiala oligosperma	CBS 342
Exophiala pisciphila	ATCC 26438
Exophiala salmonis	ATCC 16986
Exserohilum mcginnisii	R-4112
Filobasidium capsuligenum	ATCC 22179
Fonsecaea pedrosoi	CBS 271.37
Geomyces lucifugus	R-4246
Geomyces pannorum (Pseudogymnoascus pannorum)	ATCC 11501
Geosmithia argillacea (Rasamsonia argillacea)	CBS 342
Geotrichum fragrans	ATCC 11523
Gymnascella dankaliensis (Gymnoascus dankaliensis)	ATCC 22221
Histoplasma capsulatum (Ajellomyces capsulatus)	ATCC 11407
Histoplasma capsulatum var. duboisii	R-4371
Hortaea werneckii	ATCC 201677
Humicola grisea var. grisea	ATCC 22726
Hypocrea lixii (Trichoderma harzianum)	MYA-2453
Inonotus tropicalis	R-4465
Isaria javanica	CBS 134.22
Kluyveromyces marxianus	CBS 712
Lanspora coronata	ATCC 3564
Lecythophora hoffmannii (Coniochaeta hoffmannii)	ATCC 875
Lecythophora lignicola (Coniochaeta lignicola)	CBS 267.33
Lecythophora mutabilis (Coniochaeta mutabilis)	ATCC 42792
Lewia infectoria	MYA-2293[2]
Lodderomyces elongisporus	CBS 2605
Macrophomina phaseolina	ATCC 201720
Malbranchea gypsea	ATCC 34532
Malassezia furfur	ATCC 44342
Malassezia pachydermatis	ATCC 14522
Microsporum canis (Arthroderma otae)	ATCC 10214
Microsporum gypseum	ATCC 11659
Microsporum praecox	ATCC 38000
Mucor circinelloides	ATCC 11010

Table 1 (Continued)

Fungal isolates	Culture collection ID
Mycoleptodiscus terrestris	ATCC 200215
Myrmecridium schulzeri	ATCC 30065
Nannizziopsis vriesii	ATCC 3421
Neosartorya fischeri	ATCC 34561
Neosartorya pseudofischeri	ATCC 200845
Neosartorya spinosa	ATCC 5434
Neotestudina rosatii	CBS 426.62
Ochroconis constricta	ATCC 11419
Onychocola canadensis (Arachnomyces nodosetosus)	ATCC 204283
Oxyporus corticola	R-3714
Oxyporus populinus	R-3716
Paecilomyces lilacinus (Purpureocillium lilacinum)	ATCC 10114
Papulaspora sepedonioides	CBS 354.76
Penicillium chrysogenum	CBS 306.48
Penicillium citrinum	CBS 139.45
Penicillium concentricum	ATCC 58613
Penicillium commune	ATCC 10428
Penicillium marneffei (Talaromyces marneffei)	ATCC 200050
Penicillium meridianum	CBS 314.67
Penicillium spinulosum	CBS 347.48
Phaeococcomyces chersonesos (Knufia petricola)	CBS 456.65
Phanerochaete sordida	ATCC 62000
Phialemonium curvatum (Phialemoniopsis curvata)	MYA-1312
Phialemonium obovatum	ATCC 200847
Phialophora lagerbergii (Phialocephala lagerbergii)	CBS 266.33
Phialophora verrucosa	ATCC 10223
Phoma cruris-hominis	CBS 377.92
Pichia anomala (Wickerhamomyces anomalus)	CBS 7073
Pichia delftensis (Kregervanrija delftensis)	ATCC 22305
Pichia farinose (Millerozyma farinose)	CBS 185
Pichia guilliermondii (Meyerozyma guilliermondii)	CBS 2083
Pichia jadinii (Cyberlindnera jadinii)	ATCC 20248
Polyporus tricholoma	MYA-2777
Pythium aphanidermatum	ATCC 34532
Pythium insidiosum	ATCC 200268
Pseudallescheria angusta	ATCC 22965
Pseudallescheria boydii	ATCC 10808
Pseudogymnoascus roseus	ATCC 12262
Pyrenochaeta mackinnonii (Nigrograna mackinnonii)	ATCC 7654
Ramichloridium apiculatum	ATCC 13211
Ramichloridium subulatum	ATCC 52456
Rhizoclosmatium sp.	CBS 432.78
Rhizomucor variabilis var. regularior	CBS 384.95
Rhizophydium brooksianum	MYA-2891
Rhizopus oryzae	ATCC 10260
Rhizopus schipperae	ATCC 204270
Rhodotorula ferulica	ATCC 76737
Rhodotorula slooffiae	CBS 5706
Saccharomyces boulardii	ATCC 32167
Saksenaea vasiformis	R-4406
Scedosporium apiospermum (Pseudallescheria apiosperma)	CBS 116941
Schizophyllum commune	CBS 341.81
Scytalidium hyalinum (Neoscytalidium hyalinum)	CBS 145.78
Spizellomyces punctatus	ATCC 48900
Sporothrix schenckii	CBS 101428
Stereum sanguinolentum	CBS 890.76
Talaromyces helicus	CBS 335.48
Trametes elegans	CBS 250.69
Trichoderma harzianum (Hypocrea lixii)	MYA-2453
Trichophyton rubrum	CBS 286.30
Trichophyton violaceum	CBS 319.31
Trichosporon asahii	ATCC 18020
Trichosporon inkin	CBS 5585
Trichosporon lignicola	CBS 219.34
Trichosporon loubieri var. loubieri	ATCC 56048
Trichosporon mucoides	ATCC 204094
Trichosporon mycotoxinovorans	CBS 9756

[1]USDA Agricultural Research Service.

[2]Centraalbureau voor Schimmelcultures.

[3]American Type Culture Collection.

[4]Fungus Testing Laboratory, University of Texas Health Science Center at San Antonio, TX.

[5]University of Maine, Longcore Chytrid Laboratory.

() denotes current nomenclature.

on a separate section of the same petri dish to separate hyphae from agar material. Small amounts of residual agar, when carry-over occurred, did not inhibit downstream steps. Yeast or hyphae were then transferred to a 2.0-ml screw cap microfuge tube (Biospec, Inc., Bartlesville, OK, USA) containing 200 µl of Prepman Ultra Lysis Reagent (Applied Biosystems, Inc., Foster, CA, USA) and 100 µl (v/v) 0.5 mm glass beads (Biospec, Inc.). The tube was transferred to a heating block at 99 °C for 10 min, then agitated for 30 s at the highest speed in a mini bead beater (Biospec, Inc.), and finally transferred again to the 99 °C heating block for 10 min. The suspension was next pelleted by centrifugation for 1 min at 13 200 $\times \boldsymbol{g}$ in a microcentrifuge (Eppendorf, Inc., Westbury, NY, USA) and immediately used to set up PCR reactions, or the supernatant was removed after centrifugation and stored at −20 °C until further use.

Polymerase chain reaction (PCR)

Polymerase chain reaction primers used in this study are described in Table 2. PCR reactions were prepared

Table 2 Primer sequences.

Primer	Locus/Gene	Primer sequence	Reference
ITS-1	18S ribosomal RNA	5′-TCCGTAGGTGAACCTGCGG-3′	14,15
NL-4	28S ribosomal RNA	5′-GGTCCGTGTTTCAAGACGG-3′	
ITS-4	28S ribosomal RNA	5′-TCCTCCGCTTATTGATATGC-3′	14
NL-1	28S ribosomal RNA	5′-GCATATCAATAAGCGGAGGAAAAG-3	15,27
BT2a	β-tubulin (*benA*)	5′-GGTAACCAAATCGGTGCTGCTTTC-3′	46
BT2B		5′-ACCCTCAGTGTAGTGACCCTTGGC-3′	
MEF11	Translation elongation factor 1 alpha (*EF1α*)	5′-AAGAAGATTGGTTTCAACCC-3′	47
MEF41		5′-GCACCGATTTGACCAGGRTGG-3′	
EF1	Translation elongation factor 1 alpha (*EF1α*)	5′-ATGGGTAAGGARGACAAGAC-3′	48
EF2		5′-GGARGTACCAGTSATCATGTT-3′	
PG3	Endopolygalacturonase	5′-TACCATGGTTCTTTCCGA-3′	49
PG2b	(*EndoPG*)	5′-GAGAATTCRCARTCRTCYTGRTT-3′	
CL1	Calmodulin (*CAL*)	5′-GARTWCAAGGAGGCCTTCTC-3	50
CL2A		5′-TTTTTGCATCATGAGTTGGAC-3	
GPD1	Glyceraldehyde-3-phosphate dehydrogenase (*GPD*)	5′-CAACGGCTTCGGTCGCATTG-3′	51
GPD2		5′-GCCAAGCAGTTGGTTGTGC-3′	
DKO239	Unscheduled meiotic gene expression (*UME6*)	5′-GTTGGGACTAGGATTGGTAAAGC-3′	52
DKO240		5′-GATGTGGAGTAGACTTGGATAATGG-3′	
URA5.F	Orotidine monophosphate pyrophosphorylase (*URA5)*	5′-ATGTCCTCCCAAGCCCTC-3′	53
URA5.R		5′-TTAAGACCTCTGAACACCGTACTC-3′	

Primer/gene/organism association: BT2 primers were used to amplify a fragment of *benA* from *Aspergillus fumigatus*, MEF primers were used to amplify a fragment of *EF1a* from *Rhizopus oryzae*, EF primers were used to amplify a fragment of *EF1a* from *Fusarium solani*, PG primers were used to amplify a fragment of *EndoPG from Alternaria alternata*, CL primers were used to amplify a fragment of *CAL* from *Pseudallescheria boydii*, GPD primers were used to amplify a fragment of *GPD* from *Bipolaris hawaiiensis*, DKO primers were used to amplify a fragment of *UME6* from *Candida albicans*, and URA5 primers used to amplify a fragment of *URA5* from *Cryptococcus neoformans*.

in a PCR clean room in which live fungal cultures or previously amplified DNA were excluded. The set-up room contained a biohazard hood, which was used to transfer 4 μl of Prepman Ultra template DNA from the previous bead-beating step to a 0.2 ml PCR tube (Eppendorf, Inc.) stored on ice. The PCR tube containing the template DNA was transferred to a PCR workstation (enclosed, HEPA-filtered, plexiglass cabinet with fluorescent and UV lighting), which had dedicated pipetters that did not leave the workstation. Barrier tips were used for all pipetting steps. PCR reactions were performed in a volume of 50 μl, and in addition to the 4 μl of template DNA, contained 5 μl of 10x PCR buffer, 1.5 μl of 10 mmol l^{-1} dNTP (Invitrogen, Inc., Carlsbad, CA, USA), 2.5 μl of 10 mmol l^{-1} stock solution of each primer (ITS-1 forward primer and NL-4 reverse primer or second locus gene-specific primers) and 0.5 U of PCR Extender polymerase (5 Prime, Inc., Gaithersburg, MD, USA). PCR reactions were performed in an Eppendorf Master Thermocycler (Eppendorf, Inc.) and run with a temperature profile of 2 min at 94 °C followed by 35 cycles of 15 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C. The 35 cycles were followed by 5 min at 72 °C. The PCR conditions for the single copy genes were identical to those described for the ITS-1 and NL-4 primers, with the exception of the primer-specific annealing temperature. Each PCR reaction was run with a positive (control DNA) and negative (No DNA) control. After the reaction was completed, a 5-μl aliquot of each PCR product was run on a 0.7% agarose gel and visualised by ethidium bromide staining to confirm amplification. A PCR reaction was considered successful if an amplicon was generated from the template preparation, or if an additional manipulation of the template, such as a second centrifugation or dilution, yielded a product. A PCR reaction that did not generate an amplicon was considered a failed attempt after three tries that utilised new template preparations. Gel results were documented with a Kodak DC 290 imaging system (Eastman, Kodak Co., Version 1.1, Rochester, NY, USA). Positive PCR products were purified with a Qiagen PCR Purification Kit (Qiagen, Inc., Valencia, CA, USA) and sent for sequencing at the UTHSCSA Advanced Nucleic Acids core facility.

Nucleotide sequencing and analysis

Each purified template was sequenced on both strands using the two flanking primers (ITS-1 and NL-4), as

well as the two internal primers ITS-4 and NL-1 (Fig. 1). Sequencing of PCR products was performed by cycle sequencing using the BigDye Terminator cycle sequencing kit (Applied Biosystems, Inc.). The sequence data were analysed and edited using DNA sequence analysis software (Sequencher, Gene Code Co., Ann Arbor, MI, USA). Each sequence was then searched using the ITS-1 and ITS-4 primer sequences to define the ITS region, and the NL-1 and NL-4 primer sequences to define the D1/D2 region. The ITS and D1/D2 sequences were then parsed from the contig and separately used to perform individual nucleotide–nucleotide searches using the BLASTn algorithm at the NCBI website (http://www/ncbi.nlm.nih.gov/BLAST/). Fungal identifications were made based on maximum identities ≥98% and query coverages ≥90% for culture collection isolates as described previously.[27] For isolates from the Fungus Testing Laboratory, the same criteria were applied, however, in many cases, morphological identification was only to the genus level. In this case, identifications were concluded to be in agreement if the genus names were the same.

Results

Universal primer selection

Identification of any unknown fungus by PCR and sequencing first requires a PCR primer pair that is capable of amplifying any isolate regardless of phylum. To determine the utility of ITS-1 and NL-4 as a universal primer pair, the ribosomal contigs of the NCBI fungal genomes represented in Table 3 were downloaded and screened for the presence of the ITS-1 and NL-4 sequences. A total of 64 species, 46 genera and 4 phyla were screened for the ITS-1 and NL-4 primer sequences. Primer search results confirmed that each genome listed in Table 3 contained all primer sequences (ITS-1, and NL-4 as well as ITS-4 and NL-1). Therefore, the ITS-1 and NL-4 combination, which flanks the ITS and D1/D2 regions, had the potential to serve as a universal primer pair for the amplification of the ITS-D1/D2 region from all the major phyla.

A representative isolate from multiple phyla was amplified with the ITS-1 + ITS-4, NL-1 + NL-4 and ITS-1 + NL-4 primer pairs (Fig. 2) to confirm that these primers yield PCR products across phyla. These PCR products were then sequenced. Representative fungi included *Pichia farinosa* (Ascomycota), *Cryptococcus neoformans* (Basidiomycota), *Rhizophydium brooksianum* (Chytridiomycota and *Mucor circinelloides* (Zygomycota). The BLASTn results for each individual sequence yielded a correct identification to the species level, with ≥99% identity for both the ITS and D1/D2 loci and a ≥99% query coverage match among base pairs.

Performance in universal primer PCR assays

The template DNA obtained from our extraction procedure for each yeast and mould isolate from various culture collections and clinical specimens was successfully amplified. A total of 519 DNA preparations (129 yeast, 390 mould), including 95 different genera and 172 species, tested for performance in the PCR assay using the ITS-1 + NL-4 primer combination generated an amplification band using one or both of the *Taq* polymerases. For all 519 strains used in this study, we compared the top BLASTn hits from Genbank for both the ITS and D1/D2 regions for each sequence to determine fungal identity and found that the Genbank identities matched the sequence identities with a

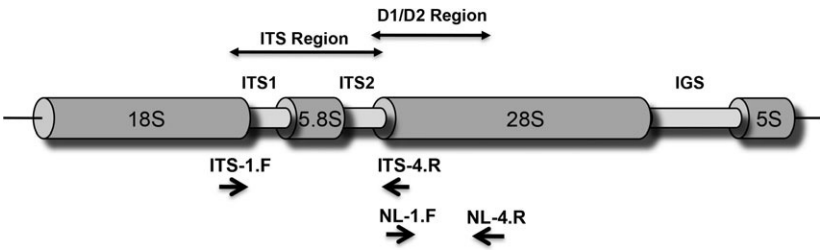

Figure 1 Polymerase chain reaction (PCR) and sequencing primer sites. Ribosomal DNA target locus of the ITS and NL primers. The ITS-1 (designated ITS-1.F, for forward primer) and NL-4 (designated NL-4.R for reverse primer) primers yield partial sequence from the 18S ribosomal subunit, complete sequence from the ITS1, 5.8S subunit, and ITS2 regions, as well as partial sequence corresponding to the D1/D2 region of the 28S ribosomal subunit. Primer sites are indicated by arrowheads. Amplified regions are indicated by double arrowhead lines. Note the ITS-4 and NL-1 primers (designated ITS-4.R and NL-1.F) overlap and are partially complementary on the 5′ end of the 28S ribosomal subunit.

Table 3 Representative phyla and genera screened for universal primer selection.

Representative genera	Strain identification
Ajellomyces capsulatus	Nam1
Ajellomyces dermatiditis	CBS 178293
Alternaria brassicola	ATCC 96836
Arthroderma gypseum	CBS 118893
Ascosphaera apis	USDA-ARSEF 7405
Aspergillus clavatus	NRRL 1
Aspergillus flavus	NRRL 3357
Aspergillus fumigatus	AF 293
Aspergillus nidulans	FGSC A4
Aspergillus terreus	NRRL 255
Blumeria graminis f. sp. Hordei	DH14
Botryotinia fuckeliana	B05.10
Candida albicans	SC5314
Candida dubliniensis	CD36
Candida glabrata	CBS 138
Candida parapsilosis	CDC 317
Candida tropicalis	MYA 3404
Chaetomium globosum	CBS 148.51
Clavispora lusitaniae	ATCC 42720
Coccidioides immitis	H538.4
Coprinopsis cinerea okayama	CBC 130
Cryptococcus neoformans	JEC21
Debaryomyces hansenii	CBS 767
Eremothecium gossypii	NRRL 7249
Fusarium oxysporum f. sp. Lycopersici	4286
Gibberella moniliformis	7600
Gibberella zeae	NRRL 2378
Glomerella graminicola	MYA 125
Grosmannia clavigera	CBS 1407
Kluyveromyces lactis	NRRL Y-1140
Kluyveromyces waltii	NCYC 2644
Lachancea thermotolerans	CBS 6340
Lodderomyces elongisporus	NRRL YB-4239
Magnaporthe grisea	NRRL 1417
Malassezia globosa	CBS 7966
Malassezia restricta	CBS 7877
Microsporum canis	CBS 113480
Nectria haematococca	NRRL 1314
Neosartorya fischeri	NRRL 181
Neurospora crassa	NRRL 2332
Paracoccidiodes brasiliensis	NRRL 2647
Penicillium marneffei	ATCC 18244
Phaeosphaeria nodorum	SN15
Phanerochaete chrysosporium	RP-78
Pichia guilliermondii	ATCC 6260
Pichia stipitis	CBS 6054
Puccinia graminis f. sp. tritici	CRL73-36-700-3
Pyrenophora tritici-repentis	PT-1C-BFP
Rhizopus oryzae	NRRL 1526
Saccharomyces bayanus	NRRL-Y 12624
Saccharomyces castelli	NRRL Y-12630
Saccharomyces cerevisiae	NRRL-Y 12632
Schizosaccharomyces japonicus	NRRL-Y 12635
Schizosaccharomyces pombe	NRRL-Y 12796
Sclerotinia sclerotiorum	CBS 1980
Talaromyces stipitatus	ATCC 10500
Trichoderma reesei	NRRL 3652
Trichophyton equinum	CBS 127.97
Trichophyton rubrum	CBS 118892
Trichophyton tonsurans	CBS 112818
Uncinocarpus reesii	CBS 1704
Ustilago maydis	CBS 521
Yarrowia lipolytica	CBS 122
Zygosaccharomyces rouxii	CBS 732

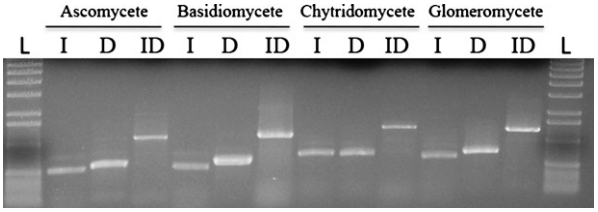

Figure 2 Amplification of template DNA from four phyla. A representative isolate from each of 4 phyla was amplified with ITS-1 + ITS-4 (I), NL-1 + NL-4 (D), or the ITS-1 + NL-4 primer pair, which combines the ITS and D1/D2 regions into a single amplicon (I + D). Band sizes ranged from ~550–700 bp to ~1.3–1.5 Kb. Representative fungi include *Pichia farinosa* (Ascomycota), *Cryptococcus neoformans* (Basidiomycota), *Rhizophydium brooksianum (Chytridiomycota)* and *Mucor circinelloides* (Zygomycota). L = 1 kb ladder.

maximum identity of 98% or greater for the culture collection isolates. An identity was also recovered from the clinical isolates using the same cut-off, which agreed with the phenotypic identity obtained from the UTHSCSA Fungus testing laboratory.

Single copy gene amplification

Template DNA from non-ribosomal genes was also tested using the same protocol to determine if the yield from the Prepman lysis protocol was sufficient for even single copy genes. The amplicons from a variety of single locus PCR reactions were run on a 0.7% agarose gel (Fig. 3) and identification of these PCR products was confirmed by a BLASTn search of the sequence. In each case, the sequence from the PCR product matched the culture and gene identity (≥99% identity).

Discussion

The frequency of human mycoses is steadily increasing, requiring the need for improved identification

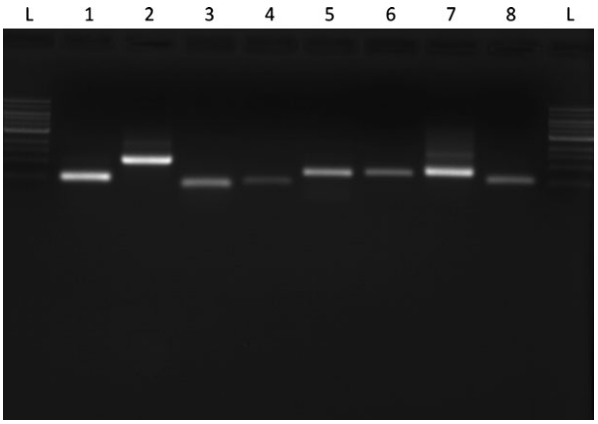

Figure 3 Amplification of single copy genes. A variety of fungal species representing multiple phyla as well as yeast and filamentous fungi were tested for amplification success using single copy targets. Lane 1, *Aspergillus fumigatus*, strain WSA-450 amplified with β-tubulin primers. Lane 2, *Cryptococcus neoformans*, strain WSA-21 amplified with *URA5* primers. Lane 3, *Rhizopus oryzae* strain WSA-299 amplified with translation elongation factor 1α primers. Lane 4, *Alternaria alternata*, strain WSA-268 amplified with Endo PG primers. Lane 5, *Fusarium solani*, strain WSA-380 amplified with translation elongation factor 1α primers. Lane 6, *Pseudallesheria boydii* strain WSA-298 amplified with calmodulin primers. Lane 7, *Candida albicans*, strain WSA-262 amplified with *UME6* primers. Lane 8. *Bipolaris hawaiiensis*, strain WSA-273 amplified with GPD primers.

methods.[11,28] Early diagnosis of certain fungal pathogens such as sterile moulds, certain *Candida* sp. and some *Penicillium* species, among others, may be difficult due to the few distinguishing morphological or biochemical features exhibited *in vitro*.[15,29]

Fungi in the phylum Zygomycota also pose a diagnostic challenge because the spectrum of opportunistic zygomycoses is expanding with more rarely seen species causing disease. In addition, the infections they cause can be fatal without rapid diagnosis and treatment due to the aggressive growth rate of this class of fungus.[30] Furthermore, fungi known to be pathogenic but not commonly seen in a clinical laboratory cannot be reliably identified without specific training or experience in mycology. Even common fungi can produce atypical phenotypes leading to misidentifications, while other fungi can take weeks to identify using culture-based methods.[31] Establishment of a non-culture-based approach for fungal identification that uses the same method regardless of species would be an important asset to the clinical microbiologist.

Molecular-based approaches are becoming more commonly used for fungal identification because they provide a more rapid and objective identification method compared to phenotype-based methods. Importantly, molecular approaches are generally not subject to phenotypic variation, which can arise due to a mutation in a gene that affects a diagnostic phenotype. In fact, sterile moulds, which do not differentiate into a distinguishable morphology due to a lack of sporulation, are generally impossible to identify in a clinical laboratory based on classical mycological methods. However, because many molecular approaches, such as sequencing, require an initial step of DNA isolation from fungi, the presence of PCR inhibitors in fungal cultures and the difficulties inherent in breaking cell walls have hindered the success of current molecular identification procedures.[10,24,32] Consequently, even though simplistic in nature, the problem of high efficiency DNA extraction from fungi is perhaps the greatest impediment to routine nucleic-acid-based identification in the clinical laboratory.

Many studies have been devoted to the development of protocols for DNA extraction from fungi. These methods have included enzymatic digestion using Proteinase K, lyticase or other cell wall degrading enzymes, physical disruption methods that include sonication, liquid nitrogen freezing and grinding of the specimen with a mortar and pestle, or bead beating.[32,33] These methods usually use a large amount of cells and utilise downstream clean-up steps, such as phenol–chloroform extractions, which can take several days as multiple additional steps such as precipitations, RNAse treatment and additional extractions may be necessary. Although these methods may generate a high yield of relatively pure DNA, high yields are of reduced significance because only very small amounts of template DNA are required for PCR-based assays. The enhanced copy number of rDNA targets further alleviates some of the yield concerns. Furthermore, because of the amplification step in PCR, multiple extraction steps carry an increased risk of contamination, and therefore, should be considered as an important template preparation criterion.

Because only small amounts of DNA are needed for PCR, numerous small yield methods have been developed. Several commercial DNA extraction kits employed for fungal DNA isolation include the Fast DNA kit (Qbiogene, Irvine, CA, USA), ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA) and the DNeasy Plant minikit (Qiagen, Inc.) to name a few.[33] All of these extraction methods are based on spin column procedures that incorporate sample lysis, removal of RNA, proteins and polysaccharides and binding of DNA to the spin column with either a solid matrix or membrane. In addition to multiple washes, each procedure requires numerous microcentrifuge

and column changes, which aside from being time consuming, can be costly.[34,35] Commercial DNA extraction kits can be expensive to use, with a cost per sample ranging from $2.56/sample to $5.93/sample. On the other hand, the PrepMan Ultra reagent that we use in our universal extraction method costs $0.53/sample. Other non-column methods, such as the Whatman FTA filter matrix (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) avoid some of the issues with columns,[9] however, this approach can be susceptible to contamination due to the need to manipulate the filter paper, but has since been improved.[36,37] In contrast, a major advantage of our strategy is that our DNA extraction method requires either one tube transfer or no tube transfers (if DNA is used immediately), thereby reducing the risk of contamination. There are also no harsh chemicals since the supernatant from the lysis step is used directly as PCR template. Our method also reduces the handling time of the fungal culture and produces enough template to yield enough PCR product to sequence from single copy targets. The timeline for the entire procedure, from lysis to sequence data, can be completed in an 8-h workday. The reduction in handling time and the small amount of sample, which can be processed in a microfuge tube, easily allows for the processing of multiple fungal strains. Importantly, prior to developing a final protocol, we attempted to prepare DNA exactly as directed in the Prepman Ultra instructions and found the results inconsistent. The key difference was vortexing vs. bead beating. Vortexing (as per instructions) generally worked for yeast, but did not work consistently for moulds. A bead beater can add capitol costs, however, single sample units are a few hundred dollars. We did not investigate vortexing with beads instead of bead beating, so this approach may be a suitable alternative to a bead beater.

Although this study focused on template preparation and PCR targets, we identified a number of enzymes that were sufficiently robust to allow amplification from a broad spectrum of fungi. Several high fidelity proofreading enzymes tested included, Phusion *Taq* (New England Biolabs, Ipswich, MA, USA), AmpliTaq DNA polymerase (Applied BioSystems, Inc.), Platinum *Taq* DNA polymerase (Invitrogen, Inc.), PCR Extender polymerase (Fisher Scientific, Inc.) and *Pfx*50 DNA polymerase (Invitrogen, Inc.). We did not identify a particular *Taq* polymerase that consistently outperformed the others, however, *Pfx*50 performed best on filamentous fungi and PCR Extender *Taq* performed best on the yeast, although overall, Extender *Taq* was most robust in our hands as it generally worked on all morphologies. We have recently tested a non-proof-reading *Taq* and found that EconoTaq (Lucigen, Inc., Middletown, WI, USA) also works well on most fungi, although yields are reduced compared to proofreading *Taq* polymerases. We did not identify any PCR enhancers (Betaine, DMSO, 2-pyrrolidone, Formamide, BSA, and DTT) that consistently yielded a significant improvement in results, so the use of enhancers was not factored into our protocol although they could improve the overall method. Instead, one of the most important factors that affected PCR success was the amount of cellular material used for the lysis step. We found that it was possible to easily over load the extraction step so that there was no PCR product. Therefore, the volume of cellular material is a critical variable and while growing isolates in a broth-based medium could make quantitation easier, we avoided broth cultures due to the need to do extra tube transfers. Unfortunately, since fungal morphology can vary between yeast and hyphae, and even these morphologies can have their own characteristics (i.e. encapsulated vs. non-encapsulated yeast), outside of starting with a relatively small inoculum (1×10^6), consistently successful protocols may need to be optimised by each laboratory. In addition, we did encounter some fungi that required more than one attempt to yield a PCR product. Problematic fungi consisted of some of the dermatiaceous fungi such as *Exophiala* sp., and some of the Mucorales. In fact, the species that regularly required multiple attempts were members of the *Apophysomyces* and *Saksenaea* genera. We also noticed that our working primer stocks had limited storage durability, leading to PCR failures, so these were made up in small aliquots and discarded when failures began to occur. In some cases, we prepared template DNA from infected tissue and utilised the VG9 primer [38] in place of ITS-1, which has 100% identity with human DNA and leads to excessive background from blood or tissue specimens. A second advantage of using VG9 is that it is possible to cover the entire ITS/D1/D2 sequence using VG9 and NL1, which overlap, and will allow sequencing through the ITS1 and NL4 boundaries using two sequences, depending on sequence length and quality. Alternatively, the Assembling the Fungal Tree of Life project has extensive primer lists <http://www.aftol.org/primers.php> if alternate primers are needed.[39,40]

For all fungi, rapidly growing cultures were the most important requirement for PCR success. Unfortunately, utilising cultures that were 2 + days old, often the age used for phenotypic identification of moulds,

was the most frequent cause of PCR failure. Consequently, our standard protocol utilised a fresh 20-h subculture as a starting point. This time frame, of course, must be balanced with yield as some fungi can grow extremely slow, particularly if they have not been subcultured.

Finally, sequence-based identification of fungi is becoming a common and frequent identification tool in mycology laboratories. We have had great success using this strategy for identifying fungi from a variety of sources including human and animal clinical isolates, environmental isolates from soil and water and even isolates from food.[27,41–45] As sequencing becomes more common as an identification tool, eventually there will be a need for standardisation, with the first step being preparing template DNA. The approach described in this study is a good starting point due to ease of use, cost and applicability.

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Conflict of interest

None.

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ARE MOLECULAR METHODS MORE SENSITIVE THAN CONVENTIONAL CULTURE FOR IDENTIFYING INVASIVE MOLDS?

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Background: Invasive fungal infections (IFI) result in significant morbidity and mortality among war-wounded and immunosuppressed individuals. Conventional methods used to diagnose IFI (namely culture and histopathology) have limitations, including reduced sensitivity and specificity. We examined the utility of a molecular (PCR)-based method for the diagnosis of IFI in both fresh and fixed tissue.

Methods: We examined paraffin-embedded tissue (subject 1) and fresh frozen (subject 2) samples from two subjects with suspected disseminated fungal infections who were either culture-negative or the culture results were not consistent with the observed histopathology. Molecular identification was performed by using a variety of PCR primer combinations directed toward the ribosomal genes. These amplicons were sequenced and assembled, then used to perform BLAST searches of Genbank using a 97% identity as a significant identity.

Results: Abbreviated history, culture, histopathology, and molecular results obtained from testing are summarized in the table below.

Subject	Brief history	Site	Conventional Culture	Histopathology	Molecular method
1	War injured with disseminated fungal infection involving the brain, abdomen, and extremities	Left triceps (ante-mortem specimen)	Pythium aphanidermatum	Septate and aseptate hyphae	Cunninghamella *sp*

1		Brain (post-mortem specimen	Not performed	Not performed	Lichtheimia *(Absidia) corymbifera*
2	Febrile neutropenic patient with suspected disseminated fungal infection	Liver	No growth	Aseptate hyphae suggestive of zygomycetes	*Rhizomucor pusillus*
2		Right upper lobe cavitary lung abscess	No growth	Aseptate hyphae suggestive of zygomycetes	*Rhizomucor pusillus*

Conclusions: This study suggests PCR-based identification and discrimination of agents of IFI is possible on both fresh and fixed tissue, and highlights the importance of initial broad empiric antifungal coverage in those suspected of having IFI. Molecular methods to diagnose IFI will likely expand our armamentarium for diagnosing IFI, and are especially useful in patients where conventional cultures are negative or are inconsistent with the results observed on histopathology.